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TITLE: Effects of CSK Homologous Kinase Overexpression on HER2/Neu-Mediated Signal Transduction Pathways in Breast Cancer Cells

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13. ABSTRACT (Maximum 200 Words)

Our proposal aims to investigate in details the potential function of CHK in breast cancer as a signal transducer in the signaling pathway from HER2/Neu receptor. The studies conducted in the period reported included: 1) the assessment of the effects of CHK on the main signaling pathways (RAFTK- and Akt-mediated) induced by the activation of HER2/Neu receptor, 2) comparison of the effects in vitro of wild-type CHK and mutant forms (enhanced/abolished binding to HER2/Neu receptor) of this CHK on the heregulin-induced activation of Src-family kinases. Our major findings are as follows: 1a) CHK directly associates with RAFTK kinase and is a negative regulator of RAFTK activation in HER2/Neu elicited transduction pathway; 1b) CHK under some circumstances facilitates activation of Akt following activation of HER2/Neu receptor; 2) the effects of CHK on Src kinase activation are directly coupled with the ability of CHK to bind the activated form of HER2/Neu receptor.

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Title: **EFFECTS OF CSK HOMOLOGOUS KINASE OVEREXPRESSION ON HER2/NEU-MEDIATED SIGNAL TRANSDUCTION PATHWAYS IN BREAST CANCER CELLS.**

P.I.: RADOSLAW ZAGOZDZON, M.D., PH.D.

INTRODUCTION: The onset of 90% of all breast cancers is random and spontaneous, while 10% of cancers have been linked to specific mutations in autosomal dominant breast cancer susceptibility genes such as BRCA1 and BRCA2. Random onset of breast cancers has in many cases been correlated with increased HER2/Neu (also termed ErbB-2) expression and Src family tyrosine kinases activity. The exact molecular mechanism of this phenomenon remains unknown. The initial steps in HER2/Neu pathways are complex, and are modulated by such processes as the autophosphorylation, cross-phosphorylation and dimerization of ErbB molecules. Furthermore ErbB cascades also interact with other signaling pathways. Association of c-Src with these receptor tyrosine kinases (PTKs) is an integral part of the signaling events mediated by the receptors, and may contribute to the malignant transformation of cells. Increased Src kinase activity observed in HER2/Neu-induced tumors results from the ability of the Src-SH2 domain to directly interact with HER2/Neu in a tyrosine phosphorylation-dependent manner. Since HER2/Neu and pp60src play a role in breast cancer and are altered during malignant transformation and tumor progression, it is important to characterize the regulation of these protein kinase activities, and the likely interactions of these kinases with each other.

Src family kinase activity is inhibited by the phosphorylation of a conserved, carboxy-terminal tyrosine. The protein tyrosine kinase responsible for this phosphorylation is Csk. We and others identified a second member of the Csk family – Csk Homologous Kinase (CHK). CHK has been suggested to have a specific role in breast cancer and the potential to be a target of breast cancer drug development. CHK, which is specifically expressed in primary breast cancer specimens, but not in normal breast tissues, phosphorylates Src and down-regulates its activity. Previous biochemical data also suggested that CHK acts as a negative growth regulator of human breast cancer. Furthermore, the interaction between the CHK-SH2 domain and $pTyr^{1248}$ of the HER2/Neu receptor is specific and critical for CHK function.

BODY:

The results mentioned above lead us to hypothesize that: (i) CHK is able to antagonize the growth-promoting signals that are mediated by HER2/Neu and Src kinases; (ii) enhancement of the binding affinity of CHK to the HER2/Neu protein might further increase the antitumor effects of CHK in breast cancer cells; (iii) peptide derived from an enhanced-binding mutant of CHK may retain its inhibiting ability on HER2/Neu and Src-mediated signaling. Therefore, the goals of this project are: (1) to investigate the effects of CHK on downstream signaling from the HER2/Neu receptor; (2) to assess the possibility of enhancing the inhibitory effects of CHK on HER2/Neu-mediated signaling; (3) to test the anti-tumor effects of CHK-derived peptides designed to diminish the transformation potential of HER2/Neu.

1a. Our recent observations indicated that RAFTK (also termed PYK2 and CAK-β) participated in MAPK-mediated intracellular signaling upon heregulin (HRG) stimulation and promoted breast carcinoma invasion. Analyses of the members of the HRG-stimulated complex revealed that RAFTK is associated with p190 RhoGAP (p190), RasGAP (modifying the MAPK transduction pathway) and ErbB-2, and plays an essential role in mediating the tyrosine phosphorylation of p190 by Src. Furthermore, studies from our group indicate that the Csk homologous kinase (CHK), a member of the Csk family, directly associates with HER2/Neu and down-regulates HER2/Neu-mediated Src kinase activation in breast cancer cells upon heregulin stimulation. Since activation of RAFTK is associated with the activity of Src family kinases, we analyzed whether CHK is capable of opposing HRG-induced activation of RAFTK. Stimulation of human T47D breast cancer cells with HRG induced the tyrosine phosphorylation of RAFTK and its association with CHK in vitro and in vivo. This interaction was mediated through the Src binding site (amino acid residue at 402) of RAFTK and the SH2 domain of CHK. RAFTK phosphorylation downstream of the activated HER2/Neu was greatly reduced in the presence of CHK. Maximal inhibition of RAFTK phosphorylation by CHK required the kinase activity of CHK. Furthermore, CHK inhibited the tyrosine phosphorylation of the focal adhesion-associated protein, paxillin, and inhibited HRG-induced T47D breast cancer cell migration. These findings indicate the role of CHK as a negative regulator in HRG- and RAFTK-mediated intracellular signaling in breast cancer cells (for details please refer to Attachment #1[1]).

1b. The serine/threonine kinase Akt has recently been the focus of intense research. Akt activation requires the phosphorylation of both Thr-308 and Ser-473. Src kinase was shown to induce activation of Akt, while Lyn kinase seems to inhibit this activation. In the present study, we investigated the effect of overexpressing the Csk homologous kinase (CHK), an inhibitor of Src-family kinases, on the phosphorylation of Akt induced by two different factors: heregulin or cisplatin. We used MCF-7 cells stably overexpressing the wild-type CHK [CHK(wt)] or dead-kinase CHK [CHK(dk)]. We observed that in MCF-7 CHK(wt) cells Lyn kinase activity was more profoundly inhibited than Src kinase activity. When the cells were stimulated with heregulin or cisplatin, Akt phosphorylation occurred more rapidly in MCF-7 CHK(wt) cells in comparison to the other clones used. Interestingly, MCF-7 CHK(wt) cells in vitro were markedly more resistant to cisplatin than the other clones used in the experiments, and surprisingly chemical inhibition of Akt phosphorylation did not influence this resistance. In summary, our results show facilitation of Akt phosphorylation by the overexpression of CHK, and provide new insight into the putative role of CHK in human cancer. (for details please refer to Attachment #2, [2])

2. The interaction between the CHK SH2 domain and Tyr(P)(1248) of the ErbB-2 receptor has been shown to be specific and critical for CHK function. In our studies, we investigated whether the interaction of the CHK SH2 domain and ErbB-2 is directly related to the inhibition of heregulin-stimulated Src kinase activity. We constructed three CHK SH2 domain binding mutants: G129R (enhanced binding), R147K (inhibited binding), and R147A (disrupted binding). NMR spectra for the domains of each construct were used to evaluate their interaction with a Tyr(P)(1248)-containing ErbB-2 peptide. G129R showed enhanced binding to ErbB-2, whereas binding was completely disrupted

by R147A. The enhanced binding mutant showed chemical shift changes at the same residues as wild-type CHK, indicating that this mutant has the same binding characteristics as the wild-type protein. Furthermore, inhibition of heregulin-stimulated Src kinase activity was markedly diminished by R147A, whereas G129R-mediated inhibition was stronger as compared with wild-type CHK. These results indicate that the specific interaction of CHK and ErbB-2 via the SH2 domain of CHK is directly related to the growth inhibitory effects of CHK. These new CHK high affinity binding constructs may serve as good candidates for inhibition of the ErbB-2/Src transduction pathway in gene therapy studies in breast cancer (for details please refer to Attachments #3[3]).

KEY RESEARCH ACCOMPLISHEMENTS:

Our major findings are as follows:

1a) CHK directly associates with RAFTK kinase and is a negative regulator of RAFTK activation in HER2/Neu elicited transduction pathway;

1b) CHK under some circumstances facilitates activation of Akt following activation of HER2/Neu receptor;

2) the effects of CHK on Src kinase activation are directly coupled with the ability of CHK to bind the activated form of HER2/Neu receptor.

REPORTABLE OUTCOMES (please see the papers attached):

1. McShan, G.D., R. Zagozdzon, S.Y. Park, et al., *Csk homologous kinase associates with RAFTK/Pyk2 in breast cancer cells and negatively regulates its activation and breast cancer cell migration.* Int J Oncol, 2002. **21**(1): p. 197-205.
2. Zagozdzon, R., C. Bougeret, Y. Fu, et al., *Overexpression of the Csk homologous kinase facilitates phosphorylation of Akt/PKB in MCF-7 cells.* Int J Oncol, 2002. **21**(6): p. 1347-52. (Note: The article is attached in a manuscript format, as the journal format is currently unavailable to us)
3. Kim, S., R. Zagozdzon, A. Meisler, et al., *Csk homologous kinase (CHK) and ErbB-2 interactions are directly coupled with CHK negative growth regulatory function in breast cancer.* J Biol Chem, 2002. **277**(39): p. 36465-70

CONCLUSIONS:

Our recent results confirm the notion that CHK is a signal transduction modulator following activation of HER2/Neu receptor in breast cancer cells. We are continuing our studies according to the previously approved Statement of Work.

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Csk homologous kinase associates with RAFTK/Pyk2 in breast cancer cells and negatively regulates its activation and breast cancer cell migration

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Abstract. Our recent observations indicated that RAFTK (also termed Pyk2 and CAK-ß) participated in intracellular signaling upon heregulin (HRG) stimulation and promoted breast carcinoma invasion. Furthermore, studies from our group indicate that the Csk homologous kinase (CHK), a member of the Csk family, directly associates with HER2/Neu and down-regulates HER2/Neu-mediated Src kinase activation in breast cancer cells upon heregulin stimulation. Since activation of RAFTK is associated with the activity of Src family kinases, we analyzed whether CHK is capable of opposing HRG-induced activation of RAFTK. Stimulation of human T47D breast cancer cells with HRG induced the tyrosine phosphorylation of RAFTK and its association with CHK *in vitro* and *in vivo*. This interaction was mediated through the Src binding site (amino acid residue at 402) of RAFTK and the SH2 domain of CHK. RAFTK phosphorylation downstream of the activated HER2/Neu was greatly reduced in the presence of CHK. Maximal inhibition of RAFTK phosphorylation by CHK required the kinase activity of CHK. Furthermore, CHK inhibited the tyrosine phosphorylation of the focal adhesion-associated protein, paxillin, and inhibited HRG-induced T47D breast cancer cell migration. These findings indicate the role of CHK as a negative regulator in HRG- and RAFTK-mediated intracellular signaling in breast cancer cells.

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Abbreviations: CHK, Csk homologous kinase; EGF, epidermal growth factor; FAK, focal adhesion kinase; GAP, GTPase activating protein; HRG, heregulin; MAP, mitogen-activated protein; PAK1, p21 activated kinase 1; PI-3 kinase, phosphatidylinositol-3 kinase; RAFTK, related adhesion focal tyrosine kinase; SH1, Src homology 1 domain; SH2, Src homology 2 domain; SH3, Src homology 3 domain; Tc, tetracycline

Key words: breast cancer, migration, CHK, RAFTK/Pyk2, heregulin

Introduction

Focal adhesion and dynamic changes in actin cytoskeleton are involved in cell growth, shape and in tumor invasion. Recently, a new family of protein tyrosine kinases (including FAK and RAFTK) was identified as the focal adhesion kinase family (reviewed in ref. 1). FAK is a widely expressed non-receptor tyrosine kinase, which participates in integrin-mediated signal transduction and regulatory pathways governing cellular functions such as adhesion, motility, anchorage-independence, and the G1 to S phase transition of the cell cycle (2-4). RAFTK (also known as Pyk2 and CAK-ß) shares 48% homology with FAK, but lacks transmembrane and myristylation domains, as well as SH2 and SH3 domains (5). Like FAK, RAFTK possesses a predicted proline-rich region in its C-terminal. RAFTK is implicated, along with pp60 src, in the coupling of some G-protein linked receptors to the MAP kinase pathway (6). RAFTK also participates in stress signaling and the JNK pathway (7), as well as in T-cell receptor signaling (8), integrin-dependent and independent signaling (9-12), and megakaryocyte signal transduction (1,12).

Expression of FAK and RAFTK was observed in breast cancer cells (13). We also observed that RAFTK is tyrosine-phosphorylated upon HRG stimulation in these cells, while FAK is constitutively tyrosine-phosphorylated in the cells (13). More importantly, our recent observations indicate that RAFTK-mediated intracellular signaling upon HRG stimulation can promote breast carcinoma cell invasion (13).

Activation of RAFTK seems to rely on autophosphorylation of Tyr-402, with subsequent association with Src family kinases, which phosphorylate other tyrosine residues within RAFTK (6). The Src family of cytoplasmic tyrosine kinases plays a critical role in proliferation, cell-cell interaction, cytoskeletal organization and signaling. Src family kinase activity is inhibited by phosphorylation of a conserved carboxyl-terminal tyrosine (Tyr-527) (14). Mutation of this conserved

tyrosine residue constitutively activates c-Src and renders it oncogenic (14-16). Csk, C-terminal Src kinase, phosphorylates the conserved carboxyl-terminal tyrosine (Tyr-527) of c-Src, suppressing c-Src kinase activity (17-23). Csk has been shown to be involved in the negative regulation of the kinase activity of Src family members *in vivo* (24,25). We and several groups identified a second member of the Csk family, known as CHK (Csk homologous kinase) (26-31). Both Csk and CHK kinases phosphorylate the conserved inhibitory tyrosine of Src family kinases, thereby repressing their kinase activity. CHK was comparable to Csk in its ability to down-regulate the *in vivo* activity of the Src family kinases, Fyn and c-Src (32-34).

Previously, we have demonstrated a specific interaction of CHK with the HER2/Neu growth factor receptor, mediated by the SH2 domain of CHK (35-37). This interaction was recently found to be mediated by Tyr 1253, the autophosphorylation site of rodent Neu homologous to the site Tyr 1248 in human HER2/Neu. This site confers oncogenicity and transforming abilities to the receptor (38-40). Additionally, CHK down-regulated the ErbB-2-mediated activation of Src family tyrosine kinases and elicited an antiproliferative effect (38). The involvement of CHK in the ErbB-2-mediated signaling pathway led us to investigate whether CHK participates in heregulin (HRG)-induced breast cancer cell migration and whether it is capable of modulating the RAFTK-mediated signal transduction.

In this study, we demonstrated a novel interaction between CHK and RAFTK upon HRG stimulation in breast cancer cells. This interaction was mediated through the Src-binding site (amino acid residue at 402) of RAFTK and the SH2 domain of CHK. We also demonstrated that CHK inhibited the tyrosine phosphorylation of RAFTK and its catalytic activity in response to HER2/Neu-mediated signaling. Transfection studies revealed that in RAFTK-expressing breast cancer cells, CHK inhibited HRG-induced cell migration. Taken together, these results suggest a novel role for CHK as a negative regulator of RAFTK during HRG-induced signaling in breast cancer cells.

Materials and methods

Materials. Recombinant human HRG (HRG-B1, 177-244) was generously provided by Dr Mark A. Sliwkowski, Genentech (San Francisco, CA). Polyclonal antibodies to CHK (Lsk), RAFTK polyclonal antibodies (RAFTK), goat anti-Pyk2, and GST antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal phosphotyrosine antibody (4G10) was kindly provided by Dr Brian Druker (Oregon Health Sciences Center). Monoclonal phosphotyrosine antibody (PY20) was purchased from Transduction Laboratories (Lexington, KY). M2 monoclonal antibodies specific for the Flag epitope were obtained from Sigma. Reagents for electrophoresis were obtained from Bio-Rad (Hercules, CA). Chemiluminescent reagents were obtained from NEN (Boston, MA). All other reagents were purchased from Sigma (St. Louis, MO).

Cell lines and transfection. Human embryonic kidney cells (293 cells) and breast cancer cells (T47D) were obtained from ATCC (American Type Culture Collection, Rockville, MD). 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. T47D cells were maintained in RPMI media with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were transfected by the calcium phosphate method.

DNA constructs. Plasmids expressing Flag-epitope-tagged CHK (pcDNA3 Flag-CHK), pcDNA3 Flag-dead-kinase CHK (dkCHK), pcDNA3 Flag-RAFTK, Flag-RAFTK Y402F were described previously (25,34,38,40,41).

Production of GST-fusion proteins and precipitations. GST-fusion proteins of SH3-SH2-CHK, SH2-CHK, SH3-CHK, and the GST control protein were cloned into the GST-fusion expression vector pGEX (Amersham Pharmacia Biotech), and proteins were expressed and purified as previously described (36,37). Precipitation experiments used 1 mg of cell lysate and 10 µg of the indicated GST-fusion proteins.

Immunoprecipitations and antibodies. Cells, $5x10^6$ 293 or T47D, were transfected using the calcium phosphate method and were lysed 48-h post-transfection with modified RIPA buffer containing protease inhibitors (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, and 1 mM each aprotinin, leupeptin, and pepstatin). Immunoprecipitations were performed using polyclonal rabbit CHK-specific antibodies (anti-Lsk) or polyclonal goat RAFTK-specific antibodies (anti-Pyk2). Control antibodies for the immunoprecipitations were, respectively, rabbit preimmune serum (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GST polyclonal goat antibodies (Santa Cruz Biotechnology). Immunoprecipitation products were analyzed by SDS-PAGE, transferred to nitrocellulose (Bio-Rad), and immunoblotted with the above antibodies or mouse monoclonal anti-phosphotyrosine antibody (4G10, generously provided by Brian J. Druker, Oregon Health Sciences University). Bound antibodies were detected by chemiluminescence (NEN), followed by exposure to film. Stripping of blots was performed at 60°C for 30 min in stripping buffer, according to the manufacturer's protocol (Amersham Pharmacia, Piscataway, NJ).

In vitro RAFTK kinase assay. The immunoprecipitated complexes, obtained by immunoprecipitating lysates with anti-RAFTK antibodies, were washed three times with lysis buffer and twice in kinase buffer [20 mM Hepes (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM Na_3VO_4, and 20 µM ATP]. The kinase assay was initiated by incubating the immune complex in kinase buffer containing 25 µg of poly (Glu/Tyr, 4:1; 20-50 kDa; Sigma) and 5 µCi [γ-^{32}P]-ATP at room temperature for 30 min. Reactions were terminated and analyzed as described (34,37).

Migration assay. Migration was performed as described (29). Results are expressed as the percentage of migrating cells as compared with the total number of cells (cells present in all Z-sections) and represent the means with SE.

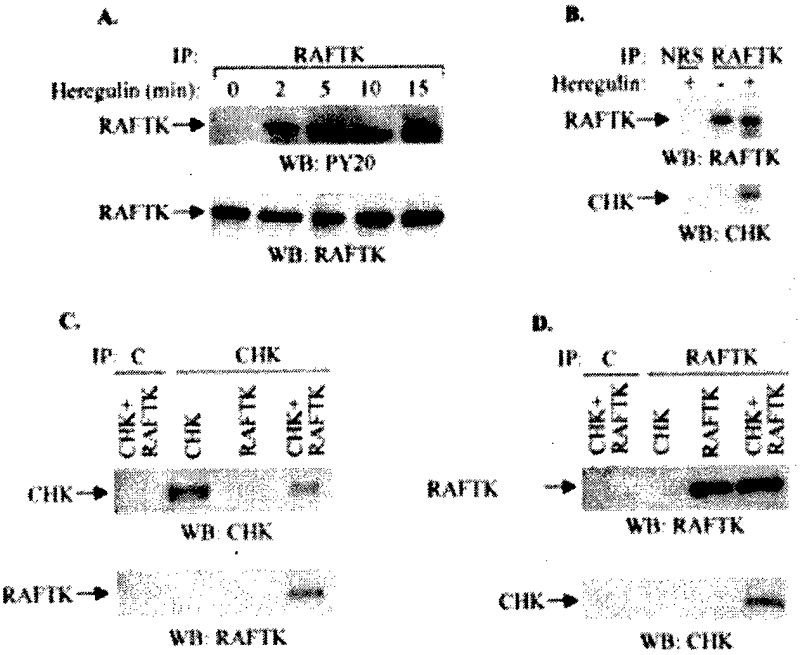

Figure 1. RAFTK association with CHK. (A), Tyrosine phosphorylation of RAFTK upon heregulin stimulation of T47D cells. Quiescent T47D cells were stimulated with 10 nM heregulin for different time periods. Cell lysates were immunoprecipitated with anti-RAFTK antibodies and separated on 7% SDS-PAGE, then blotted with PY20 anti-phosphotyrosine antibodies (upper panel). The same blot was stripped and blotted with anti-RAFTK antibodies (lower panel). (B), Association of RAFTK and CHK upon heregulin stimulation. T47D cells were transfected with pcDNA3neo-CHK. After 48 h, cells were stimulated with heregulin (10 nM for 15 min) and cell lysates were prepared and immunoprecipitated with either control rabbit serum (NRS) or RAFTK antibodies. Samples were separated on 7% SDS-PAGE and blotted with either RAFTK antibodies or CHK antibodies. (C and D), Co-immunoprecipitation of wild-type CHK and RAFTK in 293 cells. 293 cells were transiently transfected with plasmids containing full-length cDNAs encoding wild-type CHK (Flag-CHK) and RAFTK (Flag-RAFTK). Forty-eight hours post-transfection, cells were harvested and immunoprecipitated with antibody specific for CHK (C), or for RAFTK (D). Samples were separated on 10% SDS-PAGE and blotted with anti-CHK antibodies (C, upper panel) or anti-RAFTK antibodies (D, upper panel). Subsequently, the blots were stripped and reprobed with RAFTK (C, lower panel) and CHK (D, lower panel) antibodies, respectively. C, control antibody; CHK, FLAG-CHK; RAFTK, FLAG-RAFTK.

Results

Association of RAFTK with CHK in breast cancer cells. Stimulation of T47D breast cancer cells with heregulin resulted in an increase in RAFTK tyrosine phosphorylation (Fig. 1A), which remains in accordance with our recently published results (13). Enhanced tyrosine phosphorylation of RAFTK is relatively specific for heregulin stimulation, as activation by EGF did not lead to increased RAFTK tyrosine phosphorylation (data not shown). To examine CHK interaction with RAFTK in breast cancer cells, T47D cells were transfected with the Flag-CHK or pcDNA3 vector alone. Transfected cells were stimulated with heregulin and co-immunoprecipitation studies were then performed. Anti-RAFTK antibodies co-immunoprecipitated CHK from T47D cells upon heregulin stimulation, while control antibody did not co-immunoprecipitate CHK (Fig. 1B). Furthermore, in a reciprocal experiment, anti-CHK antibodies co-immunoprecipitated RAFTK from T47D cells upon heregulin stimulation (data not shown). Thus, these data suggest that CHK associates with RAFTK in breast cancer cells upon heregulin stimulation.

RAFTK and CHK specifically co-immunoprecipitate when overexpressed in 293 cells. To elucidate further the interaction of RAFTK and CHK, 293 cells were transfected with either the pcDNA3 vector alone, Flag-CHK, or Flag-RAFTK construct. Forty-eight hours following transfection, cells were harvested and 1 mg of lysate was immunoprecipitated with either control antibodies, CHK-specific rabbit polyclonal antibodies, or RAFTK-specific goat polyclonal antibodies. Immunoprecipitates were separated by 7% SDS-PAGE, transferred to nitrocellulose, and immunoblotted, as described in Materials and methods. Immunoprecipitates obtained with the CHK antibody were probed with the RAFTK antibody, which revealed that RAFTK co-immunoprecipitated with the co-transfected CHK (Fig. 1C). The reciprocal experiment, in which immunoprecipitates obtained with the RAFTK antibody were probed with the CHK antibody, revealed that CHK was present in the RAFTK-specific immunocomplexes from cells transfected with both the CHK and RAFTK cDNAs (Fig. 1D). It should be noted that CHK alone was not immunoprecipitated by the RAFTK antibody, nor was RAFTK alone immunoprecipitated by the CHK antibody. Given that immunocomplexes with either antibody were obtained only from lysates of cells transfected with both the Flag-CHK and the Flag-RAFTK cDNAs, and that control experiments using cells transfected with CHK and immunoprecipitated with RAFTK-specific antibodies or the reciprocal RAFTK cDNA transfection followed by CHK-specific immunoprecipitation

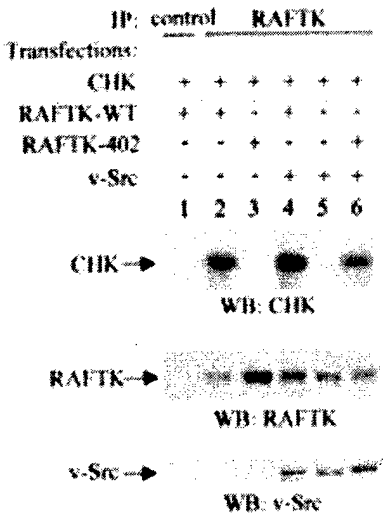

Figure 2. Maximal interaction of CHK and RAFTK requires tyrosine 402, the Src-binding site of RAFTK. T47D cells were transiently transfected with plasmids containing the full-length cDNAs encoding wild-type CHK (Flag-CHK), wild-type RAFTK (Flag-RAFTK-WT) or the Src-binding site mutant Y402F (Flag-RAFTK-402) and the constitutively active oncogenic form of Src (v-Src). Forty-eight hours post-transfection, cells were lysed and 1 mg of lysate per sample was immunoprecipitated with RAFTK-specific goat polyclonal antibody or control antibodies. Products were separated by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with CHK-specific rabbit polyclonal antibody. Subsequently, blots were stripped and reprobed with RAFTK antibody to confirm equivalent precipitation levels for the wild-type and mutant forms of RAFTK. In addition, aliquots of total cell lysates were analyzed for v-Src expression by Western blot analysis.

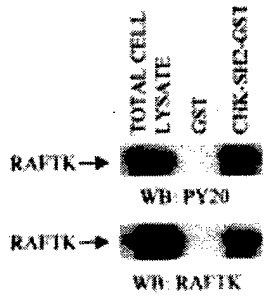

Figure 3. Interaction between RAFTK and CHK is mediated by the SH2 domain of CHK. 293 cells were transiently transfected with RAFTK expression vector. Forty-eight hours post-transfection, cells were lysed and 500 µg of lysate per sample was precipitated with CHK-SH2-GST fusion proteins or GST (control) bound to glutathione-Sepharose beads. Products were separated by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (PY20), then stripped and reprobed with anti-RAFTK antibody.

were negative, a specific interaction between the CHK and RAFTK proteins was indicated.

Maximal interaction of CHK and RAFTK requires tyrosine 402, the Src-binding site of RAFTK. In order to investigate the potential role of Src kinase in the aggregation of CHK with RAFTK, we transiently transfected T47D cells with various combinations of plasmids containing the coding sequences for wild-type CHK (Flag-CHK), wild-type RAFTK (Flag-RAFTK), Src-binding site mutant RAFTK (Flag-RAFTK-Y402F) and the constitutively active oncogenic Src (v-Src). Forty-eight hours following transfection, cells were lysed and 1 mg of lysate was precipitated with the goat polyclonal RAFTK-specific antibody or the control goat polyclonal anti-GST antibody. Following immunoprecipitation, precipitation products were separated on 7% SDS-PAGE, transferred to nitrocellulose, then immunoblotted with rabbit polyclonal CHK-specific antibody. The constitutively active, oncogenic form of Src (v-Src) enhanced the complex formation of wild-type CHK and wild-type RAFTK (Fig. 2, lane 4). No complex formation of wild-type CHK and Src-binding site mutant RAFTK (Flag-RAFTK-Y402F) was observed (Fig. 2, lane 3). Thus, the interaction of CHK with RAFTK seems to be mediated through the Src-binding site tyrosine residue 402 of RAFTK. Interestingly, v-Src partly restored the complex formation of CHK and the Src-binding mutant RAFTK (Fig. 2, lane 6).

Interaction between RAFTK and CHK is mediated by the SH2 domain of CHK. Since the tyrosine residue 402 of RAFTK is important for aggregation of RAFTK and CHK, we decided to assess whether the SH2 domain of CHK is capable of binding to RAFTK. For this purpose, we used GST pull-down assay with the use of CHK-SH2-GST fusion protein (37). 293 cells were transiently transfected with wild-type RAFTK (Flag-RAFTK) construct and 48 h following transfection cells were lysed. One mg of total cell lysate was divided into two equal parts. One part was incubated for 16 h in 4°C with CHK-SH2-GST glutathione-Sepharose beads, while the other part was incubated with GST beads as a control. Precipitates were washed three times with the lysis buffer and the proteins were separated by 7% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with antibodies against phosphotyrosine (PY20), then stripped and reprobed with antibodies against RAFTK. As shown in Fig. 3, CHK-SH2-GST strongly precipitated RAFTK from the lysate, while no precipitation was detected with GST alone. A similar pattern of precipitation was seen when we used CHK-SH2-SH3-GST fusion protein, while no precipitation of RAFTK by the CHK-SH3-GST fusion protein was seen (data not shown).

RAFTK phosphorylation downstream of the activated growth factor receptor Neu is greatly reduced in the presence of CHK. 293 cells were transiently transfected with cDNAs encoding wild-type RAFTK and activated Neu in the presence or absence of the cDNA encoding CHK. Forty-eight hours following transfection, cells were lysed and immunoprecipitated with RAFTK-specific goat polyclonal antibodies or control antibody. Products were separated by 7% SDS-PAGE, transferred to nitrocellulose, and immuno-blotted with antibodies against phosphotyrosine (4G10). Following immunoblotting with 4G10, blots were stripped and reprobed with the RAFTK antibody to confirm immunoprecipitation efficiency. Tyrosine phosphorylation and activation of wild-type RAFTK were increased in the presence of the Neu receptor (Fig. 4). Co-transfection of CHK with RAFTK and the activated Neu receptor resulted in a significant reduction of RAFTK tyrosine phosphorylation and activation (Fig. 4). In

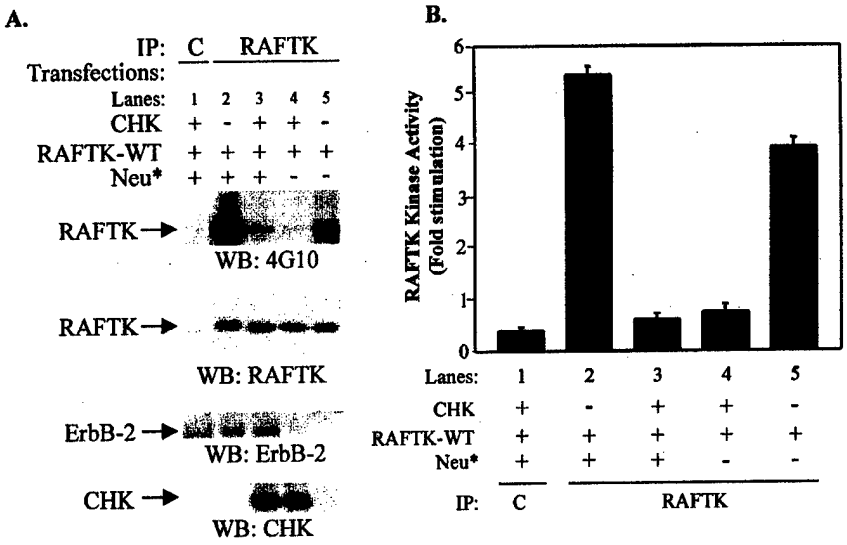

Figure 4. Co-transfection of CHK reduces the tyrosine phosphorylation of RAFTK. (A), 293 cells were transiently transfected with plasmids containing the cDNAs for wild-type RAFTK (WT), CHK and the activated neu receptor (Neu*). Forty-eight hours post-transfection, cells were lysed and 1 mg of lysate per sample was immunoprecipitated with RAFTK-specific antibody or control antibody (C). Products were separated by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (4G10). Subsequently, the blots were stripped and reprobed with the RAFTK antibody to confirm equivalent precipitation levels for all lanes. In addition, aliquots of the total cell lysates were hybridized with ErbB-2 and CHK antibodies. (B), Kinase activity of RAFTK. Total cell lysates of transfected 293 cells (as described in A) were prepared, immunoprecipitated with anti-RAFTK antibody (lanes 2-5) or with control antibody (lane 1) and then subjected to an *in vitro* kinase assay, as described in Materials and methods. C, control.

the presence of CHK without transfected activated Neu, levels of RAFTK tyrosine phosphorylation were at merely detectable levels (Fig. 4A, lane 4). Stripping and reblotting with RAFTK antibody revealed equivalent immunoprecipitation efficiency (Fig. 4A). These data indicate that the phosphorylation state of RAFTK can be negatively regulated by CHK, probably through the down-regulation of Src tyrosine kinases activated downstream of growth factor receptor activation.

CHK utilizes at least two different mechanisms to down-regulate RAFTK. To assess whether kinase activity of CHK is crucial for down-regulation of RAFTK phosphorylation, we used the kinase inactive mutant of CHK (dead-kinase CHK, dkCHK) (41). 293 cells were transfected with either the pcDNA3 vector alone, Flag-CHK, Flag-dkCHK, and/or Flag-RAFTK construct. Forty-eight hours following transfection, cells were harvested and 1 mg of lysate was immunoprecipitated with RAFTK-specific antibodies. Immunoprecipitates were separated by 7% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine PY20 antibodies. Then, the membrane was stripped and reprobed with RAFTK antibody (Fig. 5, bottom panel). Expression of CHK proteins was assessed in total cell lysates by Western blot procedure (Fig. 5, top panel). As shown in Fig. 5 (middle panel), in cells transfected both with RAFTK alone or RAFTK + pcDNA3 vector, marked phosphorylation of RAFTK was detected. Co-expression of wild-type CHK along with RAFTK strongly reduced the level of tyrosine phosphorylation of RAFTK. However, a significant decrease in RAFTK phosphorylation was also seen in the cells co-transfected with dkCHK and RAFTK, suggesting that the presence of CHK protein without kinase activity is at least partly sufficient to negatively regulate RAFTK phosphorylation.

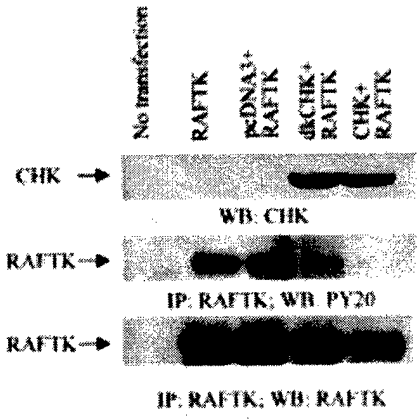

Figure 5. Effects of wild-type or dead-kinase (dk)CHK on RAFTK phosphorylation in 293 cells. 293 cells were transiently transfected with plasmids containing the cDNAs for CHK, dkCHK, and/or wild-type RAFTK. Forty-eight hours post-transfection, cells were lysed and 1 mg of lysate per sample was immunoprecipitated with RAFTK antibody. Products were separated by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (PY20) (middle panel), then stripped and reprobed with anti-RAFTK antibody (bottom panel) to confirm equivalent immunoprecipitation efficiency for all samples. Expression of CHK proteins was assessed by Western blot analysis with anti-CHK antibody (upper panel).

CHK negatively regulates paxillin phosphorylation. We have shown that paxillin is an *in vivo* binding partner and substrate for the tyrosine kinase activity of RAFTK in hematopoietic cells. In order to further evaluate the functional relevance of the interaction between CHK and RAFTK, we analyzed the phosphorylation state of paxillin in the presence of CHK, c-Src, and/or the wild-type RAFTK in comparison to paxillin

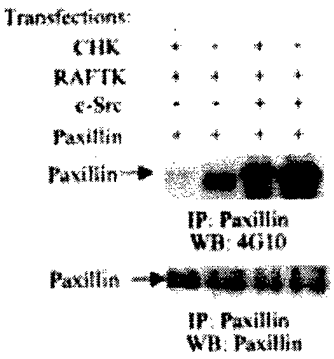

Figure 6. CHK decreases the phosphorylation of paxillin. 293 cells were transiently transfected with plasmids containing the cDNAs for CHK, c-Src, paxillin, and/or the wild-type RAFTK. Forty-eight hours post-transfection, cells were lysed and 1 mg of lysate per sample was immunoprecipitated with paxillin antibody. Products were separated by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (4G10). Subsequently, the blots were stripped and reprobed with paxillin antibody to confirm equivalent immunoprecipitation efficiency for all samples.

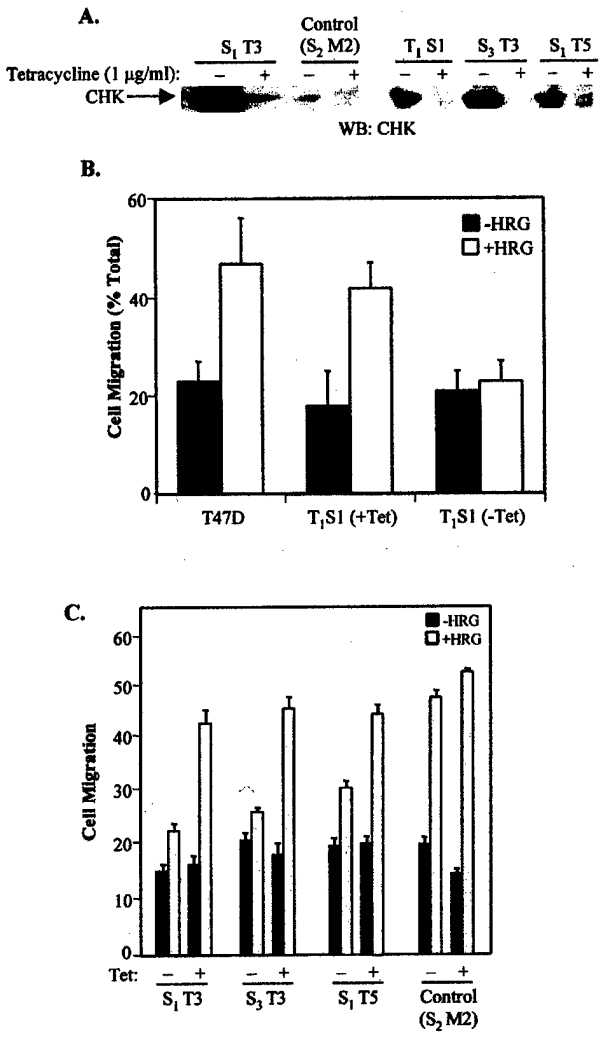

Figure 7. Inducible overexpression of CHK inhibited cell migration in T47D cells. (A), Western blot analysis of CHK protein expression in several selected clones of inducible stably transfected T47D cells expressing CHK, in the presence (+) or absence (-) of tetracycline (1 μg/ml) for 48 h. Forty μg of total cell lysates were prepared from these cell clones and examined by Western blot analysis using anti-CHK antibody. Control, T47D cells transfected with vector alone. (B and C), Effect of CHK on the migration of breast cancer cells: T47D cells or inducible stably transfected cells in the presence (+Tet) (1 μg/ml) (expressing endogenous CHK) or absence (-Tet) (overexpressing CHK) of tetracycline were untreated or treated with HRG (10 nM) for 16 h. Cell migration was then analyzed using Boyden Chambers. The data shown are mean values (± SEM) of three experiments done in triplicate. The number of migrating cells was quantitated as described in Materials and methods.

phosphorylation in the absence of CHK in transiently transfected 293 cells (Fig. 6). Tyrosine phosphorylation levels of paxillin when co-transfected with CHK and wild-type RAFTK were decreased over those obtained upon transfection of paxillin and wild-type RAFTK alone (Fig. 6, top panel, lanes 1 and 2). Further enhancement of phosphorylation was observed when c-Src was co-transfected with RAFTK-WT and paxillin. However, in the presence of CHK, the level of paxillin phosphorylation was decreased (Fig. 6, top panel, lanes 4 and 3, respectively).

Overexpression of CHK inhibited the migration of breast cancer cells in response to HRG. We have shown recently that expression of RAFTK enhances HRG-stimulated breast cancer cell invasion (13). To elucidate the effects of CHK on the HRG-induced cell migration of RAFTK-expressing breast cancer cells, inducible CHK stably transfected T47D cells were generated and characterized (Fig. 7A). Inducible stably transfected T47D cell clones, in the presence of tetracycline (expressing endogenous CHK) or in the absence of tetracycline (thus overexpressing CHK), as well as control T47D cells, were treated with HRG (10 nM) for 16 h. Cell migration was then analyzed using Boyden Chambers. The migration of T47D cells overexpressing CHK (-Tet) was significantly inhibited in the presence of HRG (Fig. 7B and C), as compared to the control T47D cells alone (without CHK) (Fig. 7B) or cells with the endogenously expressed CHK (+Tet) (Fig. 7B and C). These results demonstrate that CHK negatively regulates the HRG-induced migration of RAFTK-expressing breast cancer cells.

Discussion

Abnormalities in the expression, structure, or activity of numerous proteins governing cell proliferation, survival, migration, and invasiveness contribute to the development and maintenance of the malignant phenotype. Well-known examples of such proteins can be found within the family of receptor tyrosine kinases and their ligands. HER2/Neu (also termed ErbB-2) is a transmembrane 185 kDa receptor with intrinsic tyrosine kinase activity. Amplification of the HER2 gene and overexpression of its product (which is detected in 10-40% of human breast tumors) induce malignant transformation of mammary cells. Recent studies suggest that HER2/Neu is a ligand orphan receptor that amplifies the signal provided by other receptors of the HER family after forming ligand-induced heterodimers with them. Ligand-dependent

activation of HER1, HER3, and HER4 by EGF or heregulin (HRG) results in HER2 activation (reviewed in ref. 42). In accordance with these observations, experiments with transgenic mice have proven heregulin to contribute to the development of mammary adenocarcinomas (43), and expression of HRG was identified to correlate with a more aggressive/invasive phenotype of breast cancer cells (44). Although HRG was reported to induce the formation of a motile actin cytoskeleton structure through PI-3 kinase and p21 activated kinase 1 (PAK1), the detailed mechanism of this HRG-induced cell migration remains unknown. Cell migration is a spatially and temporally coordinated process that is regulated by a combination of cellular responses: cell polarization, membrane extension at the leading edge of the cell, formation and turnover of cell adhesion complexes, and the contraction of the cell rear (45,46). Our recent studies showed that heregulin induced the formation of a multiprotein complex with RAFTK. The proteins participating in this complex include p190 RhoGAP, p120 RasGAP and ErbB-2 (13,29). In this complex, RAFTK played an essential role in mediating the tyrosine phosphorylation of p190 by Src, and thus in controlling the association of p190 and RasGAP. Since the small G-proteins are key mediators of cell migration, RAFTK may also play an important role in cell migration through these G-proteins. RAFTK has been reported to regulate actin-based cytoskeletal reorganization and cell adhesion in various cell types (1,4). It is conceivable that RAFTK might mediate HRG-induced cell migration through the regulation of actin-based cytoskeletal reorganization and/or cell adhesion. As activation of RAFTK remains in a close correlation with the activity of Src family kinases, we used a previously identified inhibitor of Src kinase activity, Csk homologous kinase (CHK), to oppose activation of RAFTK.

In the present study, we demonstrated that HRG induced the tyrosine phosphorylation of RAFTK and its association with CHK in breast cancer cells. The transfection studies reveal the association of CHK with RAFTK to be mediated via the Src-binding site, Tyr-402, on RAFTK, and the SH2 domain of CHK (Figs. 2 and 3, respectively). These data suggest that interaction between CHK and RAFTK is at least in part direct. However, as shown in Fig. 2, co-expression of v-Src enhanced association of CHK with wild-type RAFTK and partly restored co-immunoprecipitation of CHK by anti-RAFTK antibodies when RAFTK(Y402F) mutant was used. These results suggest also indirect interaction between RAFTK and CHK. Both RAFTK and CHK were demonstrated to bind a range of other molecules. Most probably, v-Src phosphorylates tyrosine moieties in proteins already associated with RAFTK (via other ways than binding to Tyr-402), which produces new docking sites for the CHK-SH2 domain. Therefore, our data demonstrate the possibility of both a direct and indirect association of CHK and RAFTK.

Our recently published data and the results presented in Fig. 1 demonstrate induction of RAFTK phosphorylation downstream to HER2/Neu activation. When overexpressed in 293 cells, wild-type RAFTK undergoes significant phosphorylation and activation even in the absence of Neu-mediated signaling (Figs. 3-5). These conditions are sufficient for CHK to associate with RAFTK. Nevertheless, as shown in Fig. 4, co-transfection of RAFTK with the activated Neu receptor produces further enhancement of RAFTK phosphorylation and kinase activity, confirming participation of RAFTK in Neu-mediated signaling. Importantly, association of RAFTK with CHK results in a remarkable decrease in phosphorylation (Fig. 4A) and in the catalytic activity (Fig. 4B) of RAFTK, stimulated both by overexpression and/or co-expression of Neu*.

To elucidate the role of the kinase activity of CHK in the negative regulation of RAFTK activation, we used a dead-kinase mutant of CHK. We observed, that while co-expression of dkCHK with RAFTK in 293 cells was able to markedly diminish the level of phosphorylation of RAFTK, this effect was further potentiated when wild-type CHK was used (Fig. 5). These results suggest the existence of at least two mechanisms of negative regulation of RAFTK activation by CHK. The first would be a competition reaction between CHK and Src on the 402 binding site of RAFTK, in which CHK may displace Src from Y402 of RAFTK. The second, would be the inhibition of Src-kinase activity by CHK by phosphorylation of a conserved carboxyl-terminal tyrosine (Tyr-527).

In the course of our studies on interactions between RAFTK-mediated signaling and CHK, we assessed the effects of CHK on phosphorylation of paxillin in 293 cells transfected with CHK, RAFTK, and/or c-Src DNAs. The phosphorylation of paxillin is known to be mediated by both RAFTK and c-Src. We observed a marked decrease in phosphorylation of paxillin when CHK was co-expressed either with RAFTK only or RAFTK + c-Src (Fig. 6). Thus, the effects of CHK on RAFTK phosphorylation and activation impair the downstream signaling from RAFTK.

In addition, we demonstrated that conditional overexpression of CHK inhibited the HRG-induced migration of RAFTK-expressing T47D breast cancer cells (Fig. 7). Our recent data suggest that expression of CHK markedly diminishes HRG-induced invasion of MCF-7 breast cancer cells (41), which do not express RAFTK (data not shown). Therefore, to establish the link between inhibition of the RAFTK signaling pathway by CHK and the effects of CHK on HRG-induced cell migration, further studies are necessary. We generated inducible stable transfected T47D breast cancer cells which express CHK and endogenous RAFTK. As shown in Fig. 7, CHK inhibited the migration of T47D breast cells in response to HRG.

Taken together, these results indicate for the first time that CHK interacts with and can regulate RAFTK, and reveal novel findings regarding the role of CHK as a potential mediator of cytoskeletal function as well as its involvement in the migration of breast cancer cells.

Based on previously published data (6) and our results, we have proposed a model for the molecular interaction of RAFTK, Src, and CHK (Fig. 8). HRG stimulation induces the activation of RAFTK, which leads to the autophosphorylation of the tyrosine 402. It has been shown previously that the tyrosine-phosphorylated 402 of RAFTK can bind to the SH2 domain of Src, resulting in the activation of Src (6). In addition, Src has been reported to phosphorylate the tyrosine 881 of RAFTK (47,48). Based on the conserved amino acid sequence motifs (49) and the results from the

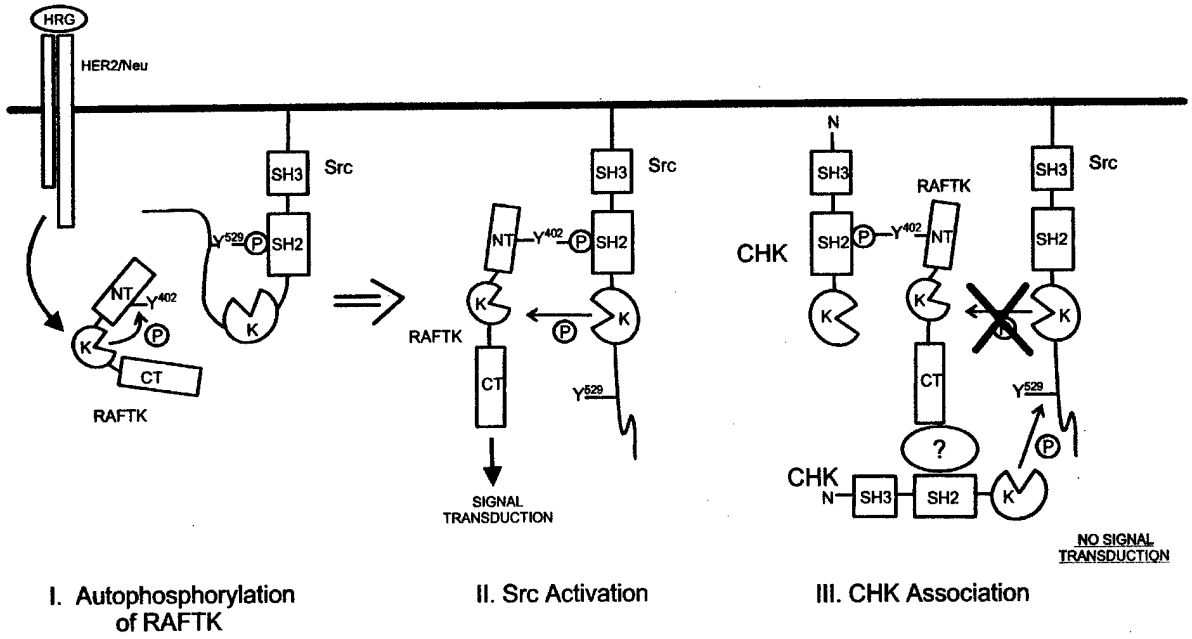

Figure 8. A proposed model for the molecular interaction of RAFTK, Src and CHK. (I), Upon activation, RAFTK autophosphorylates the tyrosine residue at 402, which is a Src-binding site. The autophosphorylated tyrosine at 402 of RAFTK binds to the SH2 domain of Src, which releases the negative regulatory binding of phospho-Tyr-529, resulting in Src activation. (II), Activated Src phosphorylates several sites of RAFTK including amino acid residues at 579, 580 and 881, which may lead to conformational changes and increases in RAFTK activity. (III), CHK associates with the activated RAFTK and inhibits its activation either by competing with Src to bind Tyr-402 or by negative regulation of Src kinase activity (CHK phosphorylates the tyrosine residues at 529 of Src, leading to the inactivation of Src). CT, C-terminal domain; K, kinase domain; NT, N-terminal domain; SH2, Src homology 2 domain; SH3, Src homology 3 domain.

study of the homologous kinase FAK (50), Src can potentially phosphorylate the tyrosine 579 and 580 of the RAFTK kinase domain. These events lead to activation of RAFTK. Our results demonstrate that co-expression of CHK leads to the negative regulation of this process by more than one mechanism.

In summary, this report demonstrates the *in vivo* association of the cytoplasmic tyrosine kinase CHK with the cytoplasmic focal adhesion-related tyrosine kinase RAFTK. This interaction results in the reduction of RAFTK tyrosine phosphorylation and catalytic activity. Furthermore, inducible overexpression of CHK down-regulated the HRG-induced migration of RAFTK-expressing cells. These findings indicate the role of CHK or its derivatives as very promising tools to target Neu-Src-RAFTK-mediated intracellular signaling in breast cancer cells.

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Overexpression of the Csk Homologous Kinase (CHK) Facilitates Phosphorylation of Akt/PKB in MCF-7 cells

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The abbreviations used are: CHK, Csk homologous kinase; Csk, C-terminal Src kinase; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; XIAP, X-linked inhibitor of apoptosis protein.

ABSTRACT

The serine/threonine kinase Akt has recently been the focus of intense research. Akt activation requires the phosphorylation of both Thr-308 and Ser-473. Src kinase was shown to induce activation of Akt, while Lyn kinase seems to inhibit this activation. In the present study, we investigated the effect of overexpressing the Csk homologous kinase (CHK)[3], an inhibitor of Src-family kinases, on the phosphorylation of Akt induced by two different factors: heregulin or cisplatin. We used MCF-7 cells stably overexpressing the wild-type CHK (CHK(wt)) or dead-kinase CHK (CHK(dk)). We observed that in MCF-7 CHK(wt) cells Lyn kinase activity was more profoundly inhibited than Src kinase activity. When the cells were stimulated with heregulin or cisplatin, Akt phosphorylation occurred more rapidly in MCF-7 CHK(wt) cells in comparison to the other clones used. Interestingly, MCF-7 CHK(wt) cells *in vitro* were markedly more resistant to cisplatin than the other clones used in the experiments, and surprisingly chemical inhibition of Akt phoshorylation did not influence this resistance. In summary, our results show facilitation of Akt phosphorylation by the overexpression of CHK, and provide new insight into the putative role of CHK in human cancer.

INTRODUCTION

The serine/threonine kinase Akt, or protein kinase B (PKB), consists of a family of highly conserved serine/threonine kinases including Akt1 and Akt2. It was originally identified as a protooncogene from rodent T cells (1). This protein has recently been a focus of intense research, as it appears to act as a transducer of signals initiated by numerous growth factor receptors as well as to lie in the crossroads of multiple cellular signaling pathways induced by other factors, e.g. DNA damaging agents (for review see ref. (2)). It is known that one of the major activities of Akt is to mediate cell survival (3), which, together with the recent discovery of the tumor suppressor PTEN as an inhibitor of Akt kinase activity (4), suggest that Akt is a critical factor in the genesis of cancer. Thus, elucidation of the mechanisms of Akt regulation as well as the physiological functions of Akt seems to be important for the understanding of cellular metabolism, apoptosis, and cancer.

To become activated, usually in a PI3-K-dependent manner, Akt requires the phosphorylation of both Thr-308 and Ser-473 (5). Recent papers show that the activity of Akt could be differentially regulated by Src-family kinases. Specifically, Akt was shown to be activated by active Src kinase (6), while Lyn kinase was demonstrated to negatively influence the function of Akt (7,8). Therefore, it can be assumed that factors modifying the activity of Src-family kinases may affect Akt activation as well.

One of the most known inhibitors of Src-family kinases is the ubiquitously expressed Csk (C-terminal Src kinase)(9). Previously, we and others have identified a cytoplasmic tyrosine kinase CHK (Csk homologous kinase) that shares about 50% homology with Csk (10). It contains SH2, SH3 and tyrosine kinase domains and is able to phosphorylate

the inhibitory C-terminal tyrosine of Src (11) and of several Src-related enzymes, including Lck (12), Fyn (13) and Lyn (11,14,15). Unlike Csk, CHK is primarily expressed solely in hematopoietic cells and in brain. Nevertheless, our studies revealed (by RT-PCR and immunohistochemistry) that the CHK protein is expressed in 70 out of 80 breast carcinoma specimens, but not in normal or benign (fibroadenoma) breast tissues (0/19) (16). Although CHK expression in primary breast tumors is very low compared to that of Csk, as it could not be detected by Western blotting and/or immunoprecipitation, the role of CHK in the development of breast cancer cannot be excluded. In our most recent studies, we have shown that stable overexpression of wild-type CHK (CHK(wt)) in MCF-7 breast cancer cells suppressed their malignant growth and was related to the inhibition of Src and Lyn protein expression and kinase activity (16). However, expression of dead-kinase mutant (CHK(dk)) did not exert that effect (16). Of note, Lyn kinase activity was more profoundly inhibited than Src kinase activity. Similarly, it was demonstrated that Csk also inactivates Lyn with a significantly higher efficiency than Src (17). In this study, we decided to assess the effect of overexpressing CHK in MCF-7 breast cancer cells on the phosphorylation of Akt induced upon stimulation with either heregulin, as a representative growth factor, or cisplatin, as one of the known DNA damaging agents. Both factors were previously shown to activate Akt (18,19). Additionally, Akt phosphorylation was recently suggested to be one of the most important factors in the resistance to cisplatin, achieved by overexpressing the X-linked inhibitor of apoptosis protein (XIAP) (20). Therefore, survival of the MCF-7 cells following the cisplatin treatment in our model was also assessed.

MATERIALS AND METHODS

Cell line and reagents. MCF-7 cells were obtained from ATCC (American Type Culture Collection, Rockville, MD). Cells were maintained in the culture medium: RPMI 1640 medium (Cellgro, Mediatech Inc., Herndon, VA) supplemented with 10% FBS (Sigma, St. Louis, MO), antibiotics, L-glutamine (2 mM), and sodium pyruvate (1 mM) (all from Cellgro). The generation of the kinase-inactivating lysine (AAG) to arginine (GCG) mutation in the CHK gene, the construction of CHK(wt)- and CHK(dk)-encoding pcDNA3 plasmids as well as stable transfections with pcDNA3neo or pcDNA3-CHK-encoding plasmids of MCF-7 cells were described in detail previously (16,21). Recombinant human heregulin (γHRG-β1, 177-244) was generously provided by Dr. Mark X. Sliwkowski (Genentech Inc., San Francisco, CA). Cisplatin was purchased from American Pharmaceutical Partners, Inc. (Los Angeles, CA). LY294002, PD98059, and Rapamycin were purchased from Cell Signaling Technology (Beverly, MA).

Western blot analysis of protein expression. For Western blot analyses, cells were scraped off the plates in cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10% glycerol) containing anti-phosphatase (1 mM sodium orthovanadate) and anti-proteases (Complete^{TM} – Protease Inhibitor Cocktail Tablets (Boehringer Mannheim GmbH, Mannheim, Germany) and lysed for 45 min at 4°C. Protein concentrations were determined using a commercial protein assay (Bio-Rad, Hercules, CA). 60 μg of total protein extracts were electrophoretically separated on 10% polyacrylamide-SDS gels, transferred to PVDF membrane, and probed with antibodies against CHK (Lsk, Santa Cruz Biotechnology, Santa Cruz, CA), Akt, and

phospho-Akt (Ser-473) (Cell Signaling Technology). Immunodetection was performed using the enhanced chemiluminescence system (NEN, Life Science Products, Inc., Boston, MA). Subsequently, blot membranes were stripped for 40 min at 60°C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and reprobed for actin (Chemicon International, Inc., Temecula, CA).

In vitro tyrosine kinase assay. MCF-7 cells were grown on 100-mm Petri dishes until 80% confluent and then total protein extracts were prepared as described above. Subsequently, 1 mg of protein was immunoprecipitated using antibodies against CHK (Lsk, Santa Cruz Biotechnology), Src (clone GD11, Upstate Biotechnology) or Lyn (Santa Cruz Biotechnology). The immunoprecipitates were washed 3 times with the lysis buffer and then resuspended in 30 µl kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mM dithiothreitol) containing phosphatase- and protease-inhibitors (see above), 0.25 mg/ml poly(Glu/Tyr)4:1 (Sigma) as an exogenous kinase substrate, 10 µM unlabeled ATP and 10 µCi of [γ-^{32}P]ATP (6000 Ci/mmol, NEN). After 10 min at 30°C, the reaction was stopped by adding SDS-sample buffer, followed by boiling the samples for 10 min. Subsequently, the samples were resolved on 12% polyacrylamide-SDS gels and the gels were stained with Coomassie blue. The labeled poly(Glu/Tyr) was excised from the gel and the radioactivity was counted.

Cell survival assay. To assess the sensitivity of MCF-7 cells to cisplatin, a standard MTT assay was used. Briefly, tumor cells were dispensed in a 96-well flat-bottomed

microtiter plate at a concentration of 1 x 10^5 cells in 200 µl. Twenty-four hours after seeding, serial dilutions of cisplatin in 50 µl of culture medium were added to the cell cultures. In the experiment using the signal transduction pathway inhibitors, PI3-kinase (LY294002), MEK1 (PD98059), or FRAP/mTOR (Rapamycin) inhibitors were added to the cell cultures at specified concentrations 1 hr prior to cisplatin treatment. After 24-hr incubation, 30 µl of 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT, Sigma) solution (3 mg/ml) was added into each well. Plates were centrifuged 4 hr later (200 *g*/ 10 min), and the supernatants were carefully removed and replaced with 200 µl of 2-propanol. After a 15-min incubation at room temperature with shaking (200 rpm), plates were read using a standard ELISA reader with 490 nm/650 nm filters. The survival of the cells was expressed as the optical density (OD 490-650) or as the relative viability (% of control cell cultures incubated with culture medium only). In the studies on the sensitivity of MCF-7 cells to a range of cisplatin concentrations, the results of the three independent experiments performed were concordant with each other. Thus, the data were pooled and expressed as a single graph. The differences in data were calculated by using the Student's *t* test. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Inhibition of Src and Lyn activities by overexpression of CHK in MCF-7 cells. In this set of experiments, we assessed the modulation of Src and Lyn kinase activities in cells expressing CHK dead-kinase (dk) or wild-type (wt) (Fig. 1A). As shown in Fig. 1B, both Lyn and Src activities were markedly lower in cells expressing CHK(wt) in comparison with all other clones. This inhibition correlated with the kinase activity of CHK (Fig. 1C). Of note, the activity of Lyn was impaired to a greater extent (60.2% inhibition vs. the parental cell line) than that of Src (38.7% inhibition). These results are concordant with our prior observations (16) Of note, the expression level of Lyn, as assessed by Western blotting, was also more deeply suppressed than the expression level of Src in cells overexpressing CHK(wt) in comparison with other clones studied (data not shown).

Kinetics of the heregulin-induced phosphorylation of Akt. MCF-7 cells, parental (MCF-7) or expressing CHK(wt), were stimulated with heregulin. Western blot analysis of the phosphorylation of Akt on Ser-473 was then performed. As shown in Fig. 2, a low level of Akt phosphorylation was already detected in MCF-7 CHK(wt) cells after 1-min incubation, while no phosphorylation of Akt could be seen in the parental cells at the same time point. Similarly, after 8 min, the phosphorylation of Akt was again more pronounced in the cells expressing CHK(wt).

Cisplatin-induced phosphorylation of Akt. As cisplatin is known to induce Akt activation, the effects of this drug in our model were studied. Four clones of MCF-7 cells:

parental (MCF-7), transfected with empty vector (neo), and expressing CHK(dk) or (wt), were stimulated with cisplatin. Next, Western blot analysis of the phosphorylation of Akt on Ser-473 was performed. Additionally, cell lysates from non-treated cells were used for Western blotting with anti-Akt antibody, and no noticeable differences were observed in terms of Akt expression between the four clones of MCF-7 cells (Fig. 3A). As shown in Fig. 3B, no phosphorylation of Akt was detected in the non-treated cells. However, in MCF-7 cells expressing CHK(wt), marked phosphorylation of Akt was already detected after 1 hr incubation, while only minor changes were observed in the other clones of MCF-7. Clearly visible changes in Akt phosphorylation were noticed in all cell types following 4 hr incubation. Nevertheless, in cells expressing CHK(wt), this phenomenon seemed to be stronger than in the other clones. Noteworthy, in cells expressing CHK(dk), the phosphorylation of Akt appeared to be even weaker than in the parental or neo MCF-7 cells.

Survival of MCF-7 cells after treatment with cisplatin and influence of signal transduction pathway inhibitors on the sensitivity of MCF-7 cells to cisplatin. To investigate the sensitivity of MCF-7 cells to cisplatin *in vitro*, different clones of the MCF-7 cells were treated with cisplatin, and cell survival was measured. As shown in Fig. 4A, the clone expressing CHK(wt) was markedly more resistant to cisplatin treatment as compared to all other clones in the experiment. Similar results were obtained with the second clone of MCF-7 cells expressing CHK(wt) (for characterization of both clones see (16)) (data not shown). Of note, the clone expressing CHK(dk) seemed to be more sensitive to cisplatin than the parental or neo MCF-7 cells. Similar differences were

also observed when the clones of MCF-7 cells were incubated with cisplatin for 48 or 72 hours (data not shown). Interestingly, no differences in sensitivity to doxorubicin or paclitaxel between the various types of MCF-7 cells were detected (data not shown).

To investigate the influence of signal transduction pathway inhibitors on the sensitivity of MCF-7 cells to cisplatin, inhibitors of PI3-kinase (LY294002), MEK1 (PD98059), or FRAP/mTOR (Rapamycin) were added to MCF-7 cell cultures 1 hour prior to 24-hour cisplatin treatment, then cell survival was measured. As shown in Fig. 4B, the inhibitors alone moderately decreased cell survival in all MCF-7 clones studied. Likewise, the inhibitors to some extent decreased the survival of cisplatin-treated MCF-7 cells (Fig. 4C). However, MCF-7 CHK(wt) cells were again significantly more resistant to cisplatin treatment. Importantly, none of the inhibitors decreased the survival of cisplatin-treated MCF-7 CHK (wt) cells to the level of the remaining MCF-7 clones, although pre-treatment with LY294002 completely abolished Akt phosphorylation in the cisplatin-treated cells (data not shown). To confirm the results obtained in MCF-7 cells, we screened several breast cancer cell lines (MDA-MB-453, BT-474, T47D, MDA-MD-361, MDA-MB-231, MCF-10A, HBL-100, SK-BR3, UACC-812) for overexpression of Src-family kinases. Although overexpression of Src kinase was quite frequent (data not shown), we did not find any other cell line in which there was a concomitant overexpression of Src and Lyn. We used stable CHK transfectants of the cell line which primarily overexpresses solely Src kinase (MDA-MB-231 cells) and we found no modulation of resistance to cisplatin in these cells by overexpression of CHK(wt) (data not shown). This result indirectly confirms the role of Lyn in response to cisplatin

treatment in breast cancer cells. Interestingly, overexpression of CHK(dk) tended to sensitize MDA-MB-231 cells to cisplatin (data not shown).

DISCUSSION

Csk Homologous Kinase (CHK) is a tyrosine kinase, sharing approximately 50% homology with Csk (10). Both kinases were shown to negatively regulate the activity of the Src-family of protein tyrosine kinases by phosphorylating their inhibitory C-terminal tyrosine (e.g. Tyr-529 in Src). Aside from that function, CHK was also shown to associate with the HER-2/neu receptor in heregulin-stimulated breast cancer cells (21,22). As both Src-family kinases and the HER2/neu receptor are closely related to the development of human cancer, the above-mentioned properties of CHK make it a putative candidate as an anti-tumor agent. Indeed, marked inhibition of *in vivo* growth of MCF-7 cells expressing CHK(wt) was observed in our recent studies (16).

Little is known about the role(s) of CHK in normal cellular physiology. It has been shown that the SH2 domain of CHK binds to several tyrosine-phosphorylated proteins that are involved in cell proliferation and differentiation, such as the activated protein tyrosine kinase receptor c-Kit in megakaryocytes (23), the activated protein tyrosine kinase TrkA in PC12 cells (24), as well as the activated protein paxillin in human blastic T cells (25). Although detailed studies on the effect of CHK on major signal transduction pathways in the cell have not been published yet, it was reported that overexpression of CHK leads to the increased phosphorylation and activation of MAP kinases in PC12 cells stimulated with NGF (24). Thus, we decided to study the effects of overexpressing CHK on the phosphorylation of Akt, the serine/threonine kinase that lies at the crossroads of multiple cellular signaling pathways. For these studies, we used previously characterized MCF-7 cells stably transfected with the genes encoding CHK(dk) and CHK(wt) (16,21).

Activation of Akt occurs, usually in a PI3-K-dependent manner, by the phosphorylation of Akt on both Thr-308 and Ser-473 (5). As no tyrosine phosphorylation has been shown to directly modulate Akt activity, the putative influence of CHK on Akt would most likely be indirect. It was already shown that Src-family kinases modulate the activity of Akt. However, the manner of this modulation is not the same for all members of the Src-family kinases. It has been shown that active Src kinase induces the activation of Akt (6). In contrast, Lyn kinase was shown in several reports to negatively regulate the activation of Akt. For instance, a several-fold increase in Ser-473 phosphorylation of Akt was reported in *lyn-/-* mast cells upon IgE receptor stimulation (7), and BCR crosslinking in Lyn-deficient B cells resulted in markedly enhanced hyperphosphorylation and activation of Akt (8). In our studies using MCF-7 cells, CHK inhibited Lyn activity to a greater extent than it did the activity of Src (Fig. 1B and (16)). Therefore, we assessed the phosphorylation of Akt in those cells stimulated with two different factors: heregulin or cisplatin. In the experiment with heregulin stimulation, two types of MCF-7 cells were used: parental and those stably transfected with CHK(wt). As shown in Fig. 2, in MCF-7 CHK(wt) cells the heregulin-induced phosphorylation of Akt occurred more rapidly than in the parental cell line, which indicates facilitation of Akt-mediated signal transduction by the overexpression of CHK(wt). To confirm this assumption, we used another agent inducing activation of Akt – cisplatin. For this experiment, four different types of MCF-7 cells were studied: parental (MCF-7), transfected with empty vector (neo), and expressing CHK dead-kinase (dk) or wild-type (wt). Again, more rapid and also stronger phosphorylation was observed in MCF-7 cells overexpressing CHK(wt) in comparison to

other cell types used in the experiment. Additionally, the phosphorylation of Akt in cells overexpressing CHK(dk) tended to be weaker than in the parental or neo cells (Fig. 3B).

As it was suggested that the Akt-mediated survival pathway may be an important contributor to cancer chemoresistance (26,27), our next step was to investigate the sensitivity of MCF-7 cells to cisplatin treatment. As shown in Fig. 4, MCF-7 cells overexpressing CHK(wt) were markedly more resistant to cisplatin than the three remaining cell types. Moreover, MCF-7 cells expressing CHK(dk) were more sensitive to cisplatin than were the parental or neo MCF-7 cells. These results correlate with the extent of Akt Ser-473 phosphorylation after cisplatin treatment (Fig. 3), suggesting that the increase in Akt activity might protect the cells from the cytotoxic influence of cisplatin. Also, these observations are in accordance with the most recent results obtained by Asselin et al. in an ovarian cancer model, as the authors of that study proposed the involvement of the Akt survival pathway in the XIAP-mediated resistance to cisplatin (20). Despite that similarity, a substantial difference exists between the influences of overexpressing XIAP versus CHK on Akt phosphorylation. XIAP caused an increase in Akt phosphorylation in quiescent cells, while overexpression of CHK in our model seemed to facilitate this process in response to other stimuli. Thus, although the co-existence of increased Akt phosphorylation and resistance to cisplatin is clear in both models, the exact mechanism of that resistance is not known and remains to be elucidated in further studies. Interestingly, inhibitors of either PI3-K-mediated (LY294002, Rapamycin) or MAPK-mediated (PD98059) pathways failed to abolish the resistance to cisplatin of the MCF-7 cells expressing CHK(wt). This observation suggests the influence of CHK overexpression on other processes than those identified to date, namely

the activation of MAP kinases (24) or the PI3-K-mediated phosphorylation of Akt as described here.

In conclusion, our results suggest that in MCF-7 cells overexpressing CHK kinase, Akt becomes more easily phosphorylated in response to growth factor (heregulin) or DNA damaging agent (cisplatin) treatment. Although CHK-mediated facilitation of Akt phosphorylation correlated with an imbalance in inhibition of Src and Lyn kinases, the cellular mechanism(s) of this phenomenon are unknown.

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apoptosis and enhances DNA repair by a common mechanism involving signaling through phosphatidyl inositol 3' kinase. Oncogene 19: 2212-2223., 2000.

FIGURE LEGENDS

Figure 1. Characterization of the expression of CHK proteins (A), and of the activities of Src, Lyn (B), and CHK (C) tyrosine kinases in MCF-7 cells.

(A) Total protein lysates were prepared from MCF-7 cells; parental (MCF-7), transfected with empty vector (MCF-7/neo), and expressing CHK (wt) or (dk). 60 μg of total protein lysates were separated on 10% SDS-PAGE and immunoblotted with Lsk antibody, then stripped and reprobed for actin as described in Materials and Methods. (B) and (C) MCF-7 cells; parental (MCF-7), transfected with empty vector (MCF-7/neo), and expressing CHK (wt) or (dk), were seeded onto 100-mm Petri dishes and grown until 80% confluence. Thereafter, cells were starved overnight in 1% FBS followed by 4 hr starvation in serum-free culture medium. The cells were then washed twice with ice-cold PBS, and total protein lysates were prepared. One mg of total lysate was immunoprecipitated with the appropriate antibodies, and an *in vitro* kinase assay using poly(Glu/Tyr) as an exogenous substrate was performed (see Materials and Methods for details).

Figure 2. Influence of incubation with heregulin on Akt phosphorylation on Ser-473 in MCF-7 cells.

MCF-7 cells; parental (MCF-7) or expressing CHK (wt), were seeded onto 100-mm Petri dishes and grown until 80% confluence. Thereafter, cells were starved overnight in 1% FBS followed by 4 hr starvation in serum-free culture medium. After this time period, heregulin was added to the culture medium at a final concentration of 100 ng/ml. Cells were incubated in the presence of heregulin for 1, 8 or 15 min. Then, cells were washed

twice with ice-cold PBS and total protein lysates were prepared as described in Materials and Methods. Sixty μg of total lysate was separated on 10% SDS-PAGE and immunoblotted with phospho-Akt (pAkt) antibody , then stripped and reprobed for actin as described in Materials and Methods.

Figure 3. Expression of Akt protein in quiescent MCF-7 cells (A) and the influence of incubation with cisplatin on the phosphorylation of Akt on Ser-473 in MCF-7 cells (B).
MCF-7 cells; parental (MCF-7), transfected with empty vector (MCF-7/neo), and expressing CHK (wt) or (dk), were seeded onto 100-mm Petri dishes and grown until 80% confluence. Subsequently, cells were starved overnight in 1% FBS followed by 4 hr starvation in serum-free culture medium. After this period, cisplatin was added to the culture medium to a final concentration of 40 μg/ml. Cells were incubated in the presence of cisplatin for 1 or 4 hours. Thereafter, cells were washed twice with ice-cold PBS and total protein lysates were prepared as described in Materials and Methods. Sixty μg of total lysate was separated on 10% SDS-PAGE and immunoblotted with Akt (A) or phospho-Akt (pAkt) antibodies (B), then stripped and reprobed for actin as described in Materials and Methods.

Figure 4. Survival of MCF-7 cells after 24-hr treatment with various concentrations of cisplatin (A), and the effects of signal transduction pathway inhibitors on the sensitivity of MCF-7 cells to cisplatin (B and C).
(A). MCF-7 cells; parental (MCF-7), transfected with empty vector (MCF-7/neo), and expressing CHK (wt) or (dk), were seeded in triplicate onto 96-well flat-bottomed

microtiter plates at a concentration of 1 x 10^5 cells in 200 µl. Twenty-four hours after seeding, serial dilutions of cisplatin in 50 µl of culture medium were added to the cell cultures. After 24-hr incubation, cell survival was assessed by MTT assay as described in Materials and Methods. Data represent the means ± SE of three independent experiments. (B) and (C). MCF-7 cells; parental (MCF-7), transfected with empty vector (MCF-7/neo), and expressing CHK (dk) or (wt), were dispensed in a 96-well flat-bottomed microtiter plate at a concentration of 1 x 10^5 cells in 200 µl. Twenty-four hours after seeding, LY294002 (final concentration of 25 µM), PD98059 (25 µM), or Rapamycin (250 nM) were added to the cell cultures. Cells were then incubated for 25 hours with the specified inhibitors (B) or one hour after the addition of the inhibitors, cisplatin was added to the cell cultures (final concentration of 80 µg/ml) and the cells were further incubated for 24 hours (C). Cell survival was assessed by MTT assay as described in Materials and Methods. Data represent the means ± SD. * $P<0.05$ vs. all other groups in the experiment.

Figure 1.

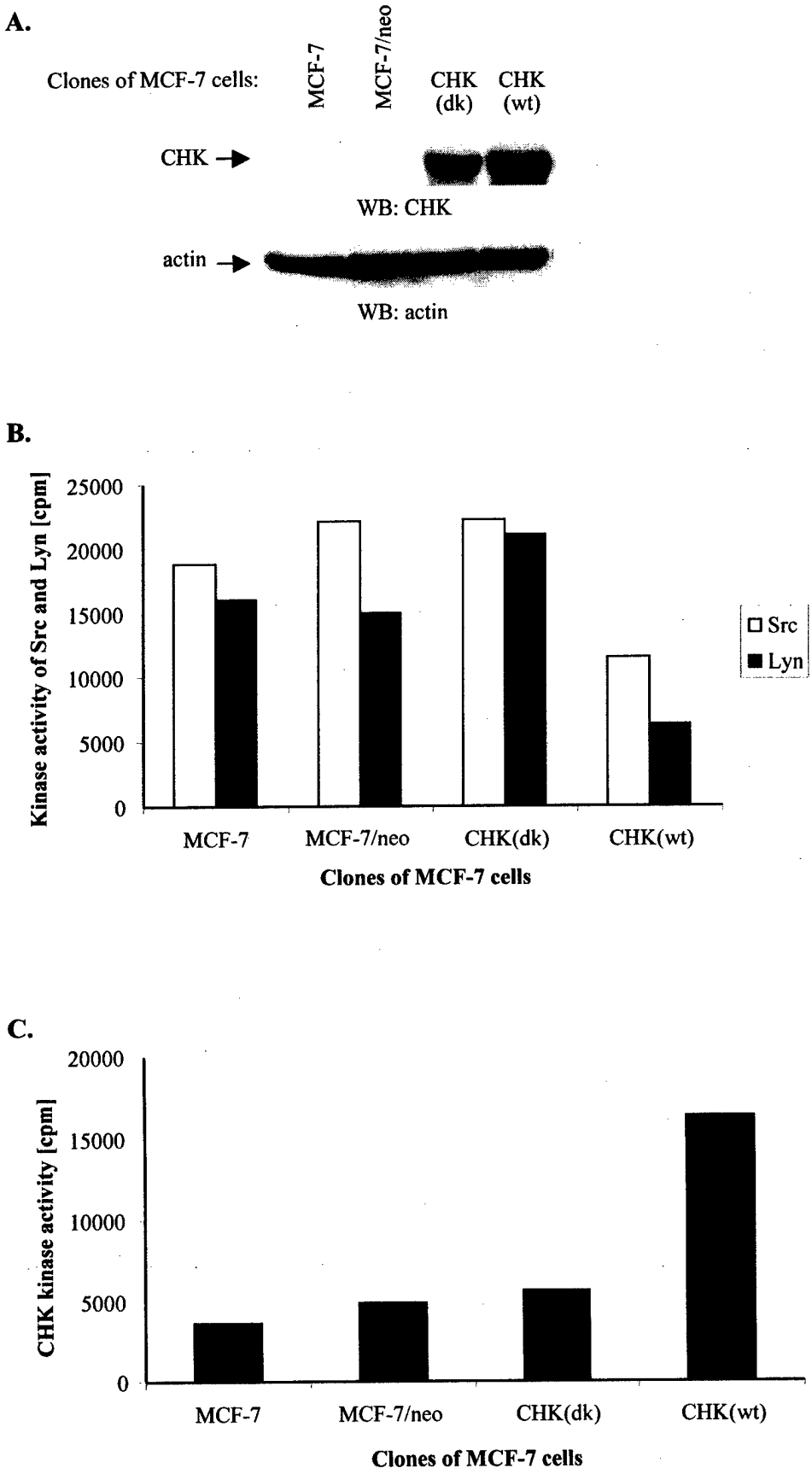

A.
Clones of MCF-7 cells:
MCF-7
MCF-7/neo
CHK (dk)
CHK (wt)
CHK
WB: CHK
actin
WB: actin
B.
Kinase activity of Src and Lyn [cpm]
25000
20000
15000
10000
5000
0
MCF-7
MCF-7/neo
CHK(dk)
CHK(wt)
Clones of MCF-7 cells
Src
Lyn
C.
CHK kinase activity [cpm]
20000
15000
10000
5000
0
MCF-7
MCF-7/neo
CHK(dk)
CHK(wt)
Clones of MCF-7 cells

Figure 2.

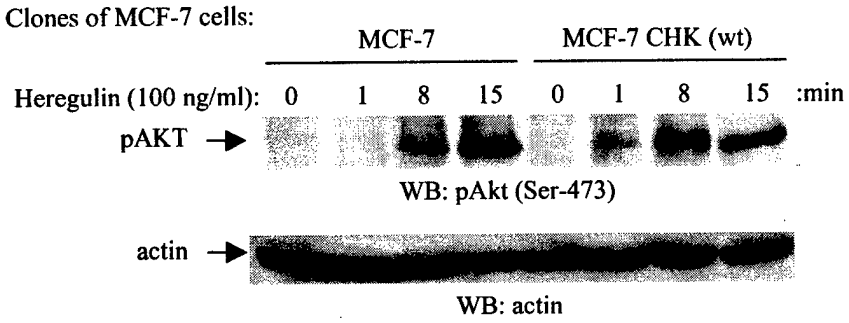

Clones of MCF-7 cells:
MCF-7
MCF-7 CHK (wt)
Heregulin (100 ng/ml): 0 1 8 15 0 1 8 15 :min
pAKT
WB: pAkt (Ser-473)
actin
WB: actin

Figure 3.

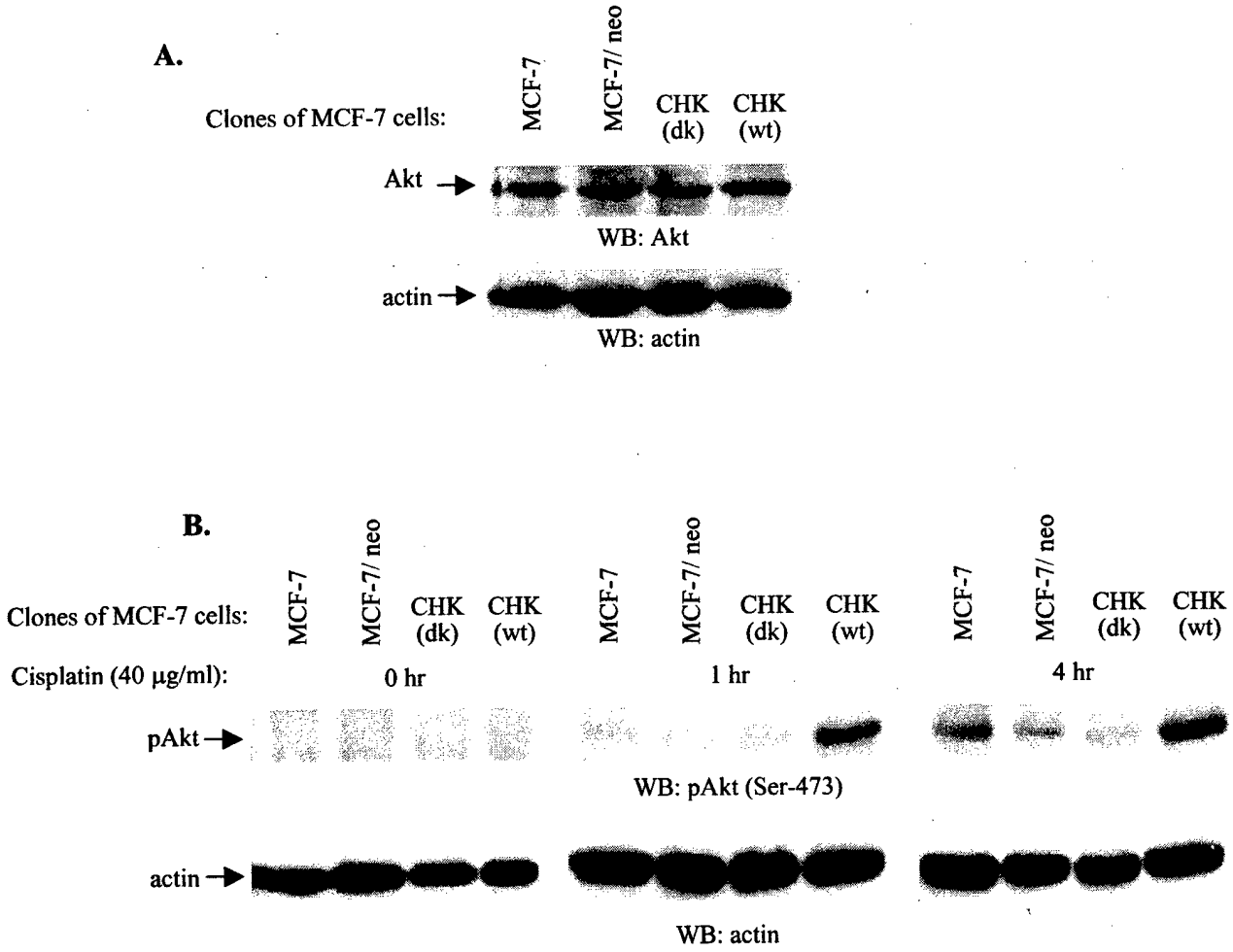
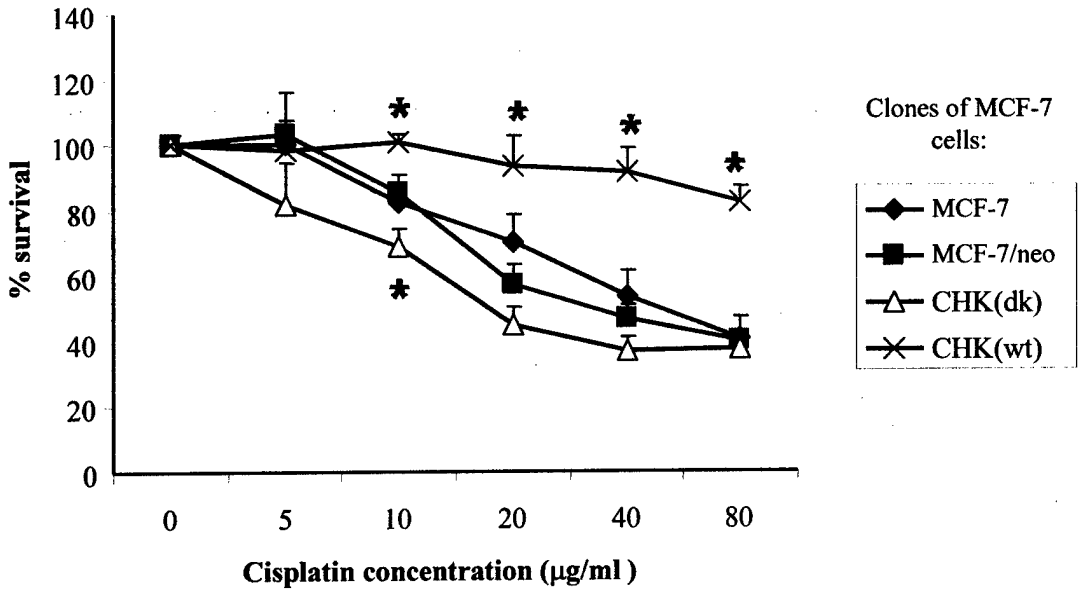
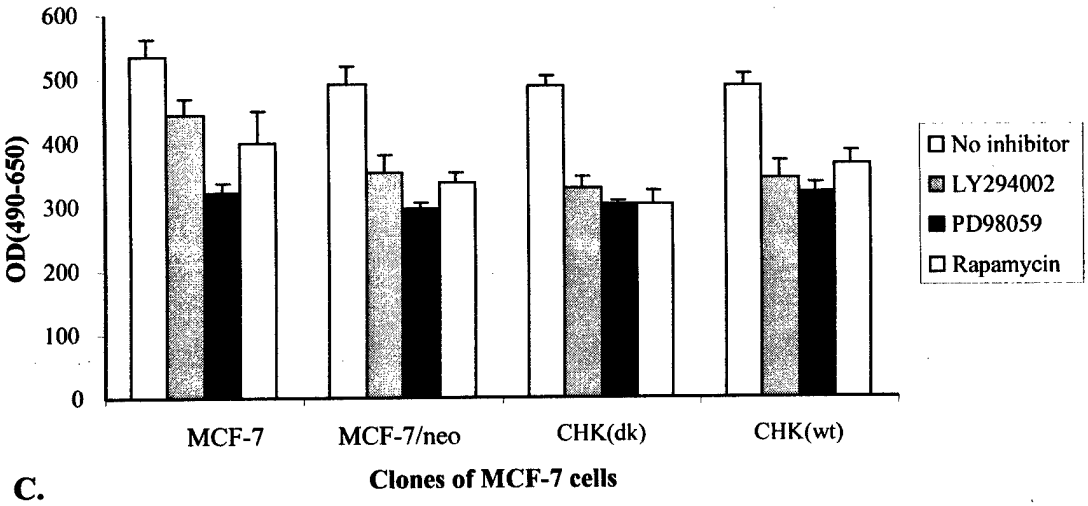

A.
Clones of MCF-7 cells:
MCF-7
MCF-7/ neo
CHK (dk)
CHK (wt)
Akt
WB: Akt
actin
WB: actin
B.
Clones of MCF-7 cells:
MCF-7
MCF-7/ neo
CHK (dk)
CHK (wt)
Cisplatin (40 μg/ml):
0 hr
1 hr
4 hr
pAkt
WB: pAkt (Ser-473)
actin
WB: actin

Figure 4.

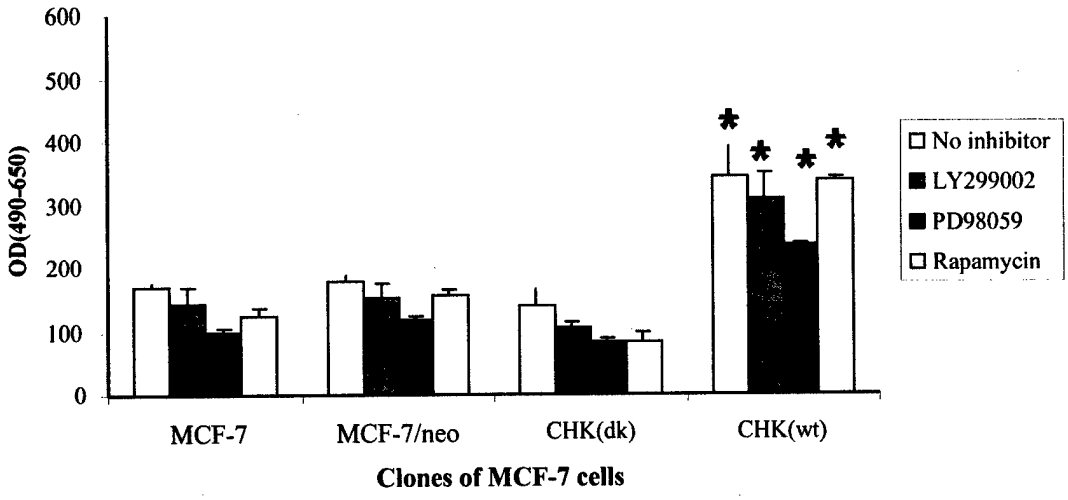
A.


140
120
100
80
60
40
20
0
% survival
0
5
10
20
40
80
Cisplatin concentration (μg/ml)
Clones of MCF-7 cells:
MCF-7
MCF-7/neo
CHK(dk)
CHK(wt)

B.


600
500
400
300
200
100
0
OD(490-650)
MCF-7
MCF-7/neo
CHK(dk)
CHK(wt)
Clones of MCF-7 cells
No inhibitor
LY294002
PD98059
Rapamycin

C.


600
500
400
300
200
100
0
OD(490-650)
MCF-7
MCF-7/neo
CHK(dk)
CHK(wt)
Clones of MCF-7 cells
No inhibitor
LY299002
PD98059
Rapamycin

APPENDIX #3

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Csk Homologous Kinase (CHK) and ErbB-2 Interactions Are Directly Coupled with CHK Negative Growth Regulatory Function in Breast Cancer*

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Our previous studies demonstrated that Csk homologous kinase (CHK) acts as a negative growth regulator of human breast cancer through inhibition of ErbB-2/neu-mediated Src family kinase activity (Bougeret, C., Jiang, S., Keydar, I., and Avraham, H. (2001) *J. Biol. Chem.* 276, 33711–33720. The interaction between the CHK SH2 domain and Tyr(P)^{1248} of the ErbB-2 receptor has been shown to be specific and critical for CHK function. In this report, we investigated whether the interaction of the CHK SH2 domain and ErbB-2 is directly related to the inhibition of heregulin-stimulated Src kinase activity. We constructed three CHK SH2 domain binding mutants: G129R (enhanced binding), R147K (inhibited binding), and R147A (disrupted binding). NMR spectra for the domains of each construct were used to evaluate their interaction with a Tyr(P)^{1248}-containing ErbB-2 peptide. G129R showed enhanced binding to ErbB-2, whereas binding was completely disrupted by R147A. The enhanced binding mutant showed chemical shift changes at the same residues as wild-type CHK, indicating that this mutant has the same binding characteristics as the wild-type protein. Furthermore, inhibition of heregulin-stimulated Src kinase activity was markedly diminished by R147A, whereas G129R-mediated inhibition was stronger as compared with wild-type CHK. These results indicate that the specific interaction of CHK and ErbB-2 via the SH2 domain of CHK is directly related to the growth inhibitory effects of CHK. These new CHK high affinity binding constructs may serve as good candidates for inhibition of the ErbB-2/Src transduction pathway in gene therapy studies in breast cancer.

The majority of breast carcinomas appear to be sporadic and have a complex accumulation of molecular and cellular abnormalities that constitute the malignant phenotype (1, 2). In many cases, the random onset of breast cancer correlates with overexpression of the ErbB-2/neu receptor and Src tyrosine kinase activity (3, 4). Downstream activation by the ErbB-2/neu receptor involves intracellular pathways mediated by Ras/mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and phospholipase Cγ; however, the molecular mechanisms of these processes are poorly understood (5). Src tyrosine kinase has been suggested to be a main downstream activator of the ErbB-2/neu receptor because the increased Src kinase activity observed in ErbB-2/neu-induced tumors results from the ability of the Src SH2 [1] domain to interact directly with ErbB-2/neu in a phosphotyrosinedependent manner (6, 7). Once the ErbB-2/neu receptor is activated by heregulin, it undergoes autophosphorylation at five tyrosine residues located in its non-catalytic carboxyl terminus. The autophosphorylation of ErbB-2/neu can also be induced in the absence of any ligand by high level overexpression of ErbB-2/neu protein (8), as occurs in BT474 or MDA-MB-361 cells.[2] The autophosphorylated tyrosine residues provide docking sites for proteins to connect to intracellular pathways (9, 10). The individual target and effect of each phosphotyrosine are not clear, but an add-back mutation study showed that autophosphorylation of tyrosine residues is involved in both the positive and negative effects on ErbB-2/neu-mediated transformation (11). Tyr^{1248} of ErbB-2/neu, which is conserved between human and rodent ErbB-2/neu, has been suggested to be the most critical residue for the oncogenicity of the constitutively activated receptor (12). Thus, the study of proteins that bind to Tyr(P)^{1248} of ErbB-2/neu is important in elucidating ErbB-2/neu-mediated signaling and function in cancer development.

The Csk homologous kinase (CHK) protein comprises SH3, SH2, and tyrosine kinase domains. Its SH2 domain interacts with Tyr(P)^{1248} of the ErbB-2/neu receptor in a ligand- and receptor-specific manner (13). CHK, like Csk, down-regulates Src kinase activity by phosphorylation of the conserved tyrosine residue in the carboxyl terminus of Src-related enzymes *in vitro*. However, CHK has been suggested to play a specific role as a novel negative growth regulator of human breast cancer on the basis of the following observations. 1) Unlike Csk, which is ubiquitously expressed and cannot associate with ErbB-2, CHK is specifically expressed in primary breast cancer specimens, but not in normal breast tissues (13–15). CHK expression in normal tissues is restricted to hematopoietic cells and brain (16–18). 2) CHK binds directly to Tyr(P)^{1248} of the ErbB-2/neu receptor kinase upon heregulin stimulation and inhibits Src

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This paper is dedicated to Charlene Engelhard for her continuing friendship and support of our research program.

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[1] The abbreviations used are: SH, Src homology; CHK, Csk homologous kinase; GST, glutathione *S*-transferase; EGFP, enhanced green fluorescent protein; HSQC, heteronuclear single quantum correlation.

[2] R. Zagozdzon and H. Avraham, unpublished data.

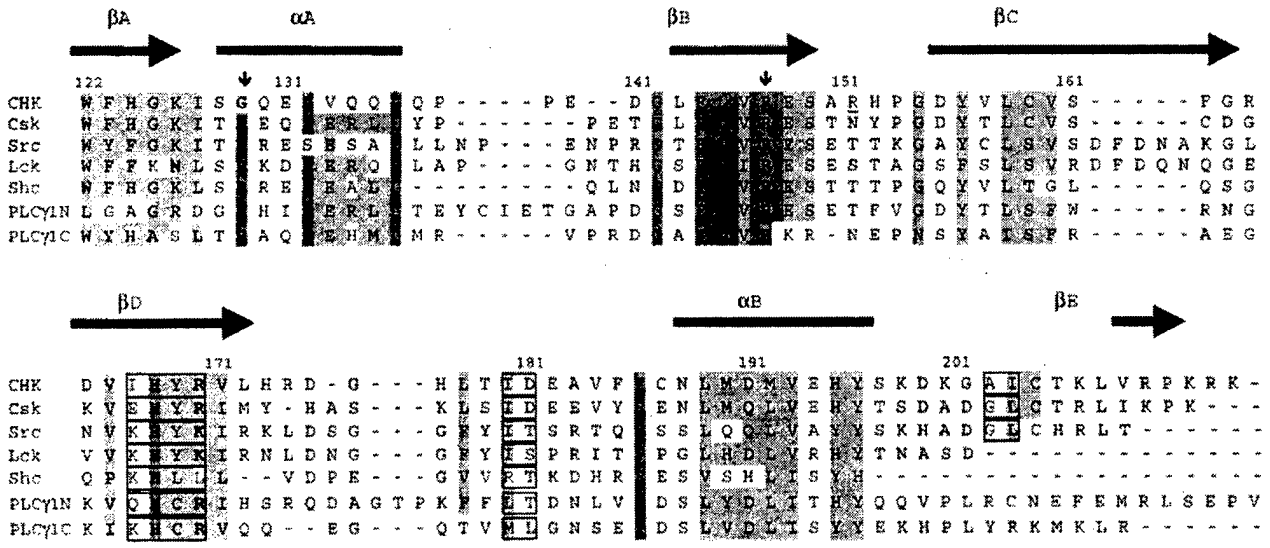

FIG. 1. **Structural alignment of several SH2 domains.** The SH2 domains of CHK, Csk, Src, Lck, Shc, and phospholipase Cγ1 (*PLCγ1*) were aligned using the T-COFFEE program (available at ch.embnet.org). *Solid bars* above the amino acid sequences indicate the secondary structural elements. Strictly conserved residues are shown in *dark gray*, and moderately conserved residues are shown in *light gray*. Residues involved in the interaction with Tyr(P) are shown in *boldface*, and residues that contribute to the interactions in the region C-terminal to Tyr(P) are *boxed*. The mutation sites (Gly^{129} and Arg^{147}) are indicated by *vertical arrows*. The nonconserved Arg^{151} is *underlined*.

kinase activity (17). Substantial evidence supports a role for CHK as a negative growth regulator of human breast cancer through inhibition of ErbB-2/neu-mediated Src family kinase activity. Overexpression of CHK in MCF-7 breast cancer cells markedly inhibits the cell growth, transformation, and invasion induced by heregulin and also causes a significant delay of cell entry into mitosis. Furthermore, the tumor growth of wild-type CHK-transfected MCF-7 cells in nude mice is significantly inhibited compared with that of non-transfected MCF-7 cells or cells transfected with kinase-dead CHK (18). The specific expression of CHK in breast cancer tissues and its inhibitory effect on cancer development strongly suggest the potential of the CHK protein as an anticancer drug and a target of gene therapy.

Mechanism-based target identification and structure-based drug design are promising for the development of selective anticancer drugs that would replace conventional cancer chemotherapy and its associated cytotoxic side effects (19). Precise biochemical and structural information on the CHK SH2 domain and the $Tyr(P)^{1248}$ containing peptide is necessary to develop CHK as a potential target of breast cancer therapy. We compared the primary sequence of the CHK SH2 domain with those of other SH2 domains (Fig. 1). The structures of a number of other SH2 domains and their complexes with phosphopeptides derived from biological targets were studied by NMR and x-ray crystallography. All contain similar secondary structural elements (Fig. 1) (20–22). The structures reveal general Tyr(P)-binding sites as well as specificity-determining sites in the SH2 domains. Although the mode of recognition of the cognate phosphopeptides by two types of SH2 domains is different (see "Discussion"), the geometry of the Tyr(P)-binding pocket within each SH2 domain is conserved in the SH2 domain family (23). SH2 domains display positively charged pockets lined with consensus basic residues of Arg (αA1), Arg (βB5), His (βD4), and Lys/Arg (βD6) (Fig. 1). Extensive interactions have been identified between the sequence C-terminal to Tyr(P) of the peptide and several residues in the βD strand, the βD-αB loop, and the αB-βE loop, which play a regulatory role in the specific binding. We constructed three CHK SH2 domain binding mutants in αA and βB to test the biological significance of their binding in growth inhibitory function. We also performed NMR experiments to identify, in the CHK SH2 domain, the binding sites for a phosphotyrosine-containing peptide derived from ErbB-2.

CHK has been suggested to have a specific role in breast cancer and to be a potential target for breast cancer drug development. Mutation of residues to confer modified binding to $Tyr(P)^{1248}$ of ErbB-2/neu will elicit functional insights into the binding of CHK to this receptor.

EXPERIMENTAL PROCEDURES

Materials—Recombinant heregulin-β1 (amino acids 177–244) was obtained from Genentech, Inc. (San Francisco, CA). Anti-phospho-HER2/ErbB-2 (Tyr^{1248}) antibody was purchased from Cell Signaling Technology, Inc. The primers for PCR were purchased from Integrated DNA Tech. ECL reagents were purchased from Amersham Biosciences.

Cell Lines—Three different breast cancer cell lines with various levels of ErbB-2/neu protein expression were used: MCF-7 (normal level expression), T47D (moderate level overexpression), and BT474 (high level overexpression). All three cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 medium (Cellgro, Inc.) supplemented with 10% fetal bovine serum and 3.5 μg/ml insulin (Sigma). Prior to stimulation with heregulin, cells were starved overnight in medium containing 1% fetal bovine serum and then incubated for 4 h in serum-free medium.

Peptide Synthesis and Purification—A peptide containing $Tyr(P)^{1248}$ of ErbB-2, ENPEpYLGLDV, was synthesized using solid-phase Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-based peptide synthesis with an acetylated N terminus and amidated C terminus (Tufts Core Facility, Boston, MA). All peptides were purified by C_{18} reverse-phase high performance liquid chromatography, and identities were confirmed using matrix-assisted laser desorption ionization mass spectroscopy.

Construction and Purification of the CHK SH2 Domain—The CHK SH2 domain constructs (residues 116–217: G129R, R147A, R147K, and wild-type) were subcloned into the pGEX2T vector using the restriction endonuclease sites *Bam*HI and *Eco*RI. Point mutations were generated by PCR using the QuikChange site-directed mutagenesis system (Stratagene) according to the manufacturer's instructions. Mutants were verified by sequencing. At least three independently generated mutants were tested for each construct. The glutathione *S*-transferase (GST)-fused CHK SH2 domains were expressed in bacteria (BL21(DE3) cells) and purified following published procedures (13, 14). Isolated SH2 domains were generated by thrombin cleavage, followed by purification on a benzamidine-Sepharose 6B column (Amersham Biosciences) (13, 14). Approximately 10 mg of protein from all constructs were purified from 1-liter cultures in rich medium (LB medium) and 5 mg from culture in minimal medium.

Generation of CHK-encoding pIRES2-EGFP Vectors—To investigate the effects of the generated mutants in breast cancer cells, the same mutations were generated in the full-length form of the CHK gene originating from previously described pcDNA3-based constructs (19–21). The generation and characterization of a CHK mutant lacking kinase activity were described in detail previously (15). All studied forms of the CHK gene were cloned into the pIRES2-EGFP mammalian expression vector (CLONTECH). Expression of wild-type as well as mutant CHK proteins was assessed by transient transfection of 293T cells and by Western blot analysis.

Binding of ErbB-2 to GST Fusion Proteins—T47D or BT474 cells (~5×10^6 cells/plate) were starved overnight in medium containing 1% fetal bovine serum, followed by additional starvation in serum-free medium for 4 h at 37 °C. The starved T47D or BT474 cells were then stimulated with 20 nM heregulin for 8 min at room temperature. The stimulation was terminated by the addition of ice-cold lysis buffer (0.1% SDS and 1% Triton X-100 in Tris-buffered saline containing 10% glycerol, 1 mM EDTA, 0.5 mM Na_3VO_4, and protease inhibitor mixture (Roche Molecular Biochemicals)). Lysates were precleared by centrifugation (14,000 rpm, 15 min) and incubated for 90 min at 4 °C with 10 μg of GST fusion proteins coupled to glutathione-Sepharose beads. Next, the beads were washed three times with lysis buffer. SDS buffer was then added, and samples were analyzed on an SDS-7% polyacrylamide gel. Proteins were transferred onto Immobilon-PM membranes (Millipore Corp.), and bound proteins were immunoblotted with anti-phospho-HER2/ErbB-2 (Tyr^{1248}) antibody. The blots were developed using the ECL system.

NMR Spectroscopy—^{15}N,^{13}C-Double-labeled protein samples or ^{15}N-labeled samples were obtained by growing the transformed bacteria in minimal medium containing $^{15}NH_4Cl$ and ^{13}C-labeled glucose or $^{15}NH_4Cl$ and unlabeled glucose as the sole sources of nitrogen and carbon, respectively (22–24). Protein was purified following the same procedures as described above, except that the purified proteins were concentrated using Centricon centrifugation filtration units (Millipore Corp.) with a M_r 3000 cutoff. The purified proteins were then exchanged into the final NMR sample buffer containing 50 mM phosphate (pH 7.5), 50 mM NaCl, 1 mM EDTA, and 2 mM perdeuterated dithiothreitol (Cambridge Isotope Laboratories, Cambridge, MA). Optimal conditions were predetermined using microdialysis against a variety of buffers (25–27). NMR experiments were performed on Bruker AMX 500-MHz and Avance 600-MHz spectrometers. Titration of the protein with the dissolved peptide (in the same buffer) was monitored by changes in ^{15}N-^{1}H heteronuclear single quantum correlation (HSQC) spectra collected at peptide/protein molar ratios of 0, 0.25, 0.5, 0.75, 0.85, 1.0, 1.25, 1.5, and 2.0. Dissociation constants (K_d) of peptide binding were determined by analyzing the titration data assuming fast exchange and by using CRVFIT (a nonlinear least-squares fitting program obtained from R. Boyko and B. D. Sykes). For backbone assignment of the protein, triple resonance experiments, including HNCA, HN(CO)CA, HNCACB, and HN(CO)CACB (27, 28), were performed and ^{15}N-separated three-dimensional nuclear Overhauser effect correlation and total correlation spectra were recorded. The data were processed and analyzed using FELIX 98 (Accelyrs, Inc.).

In Vitro Src Tyrosine Kinase Assay—For this experiment, MCF-7 cells were chosen because of their overexpression of Src kinase. The cells were grown on 100-mm Petri dishes until 80% confluent and then transfected with 10 μg of various CHK-pIRES2-EGFP constructs using LipofectAMINE 2000 (Invitrogen) as recommended by the manufacturer. Twenty-four hours after transfection, the cells were starved overnight in medium containing 1% fetal bovine serum, followed by additional starvation in serum-free medium for 4 h at 37 °C. The starved MCF-7 cells were then stimulated with 20 nM heregulin for 8 min at room temperature, and total protein extracts were prepared as described above. One milligram of protein was immunoprecipitated using antibodies against Src (Santa Cruz Biotechnology). The immunoprecipitates were washed three times with lysis buffer and then resuspended in 30 μl of kinase buffer (50 mM Tris-HCl (pH 7.4), 10 mM $MnCl_2$, 10 mM $MgCl_2$, 0.1% Triton X-100, and 1 mM dithiothreitol) containing phosphatase and protease inhibitors, 0.25 mg/ml poly(Glu/Tyr) (4:1; Sigma) as an exogenous kinase substrate, 10 μM unlabeled ATP, and 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; PerkinElmer Life Sciences). After 10 min at 30 °C, the reaction was stopped by adding SDS sample buffer and boiling the samples for 10 min. Subsequently, the samples were resolved on SDS-12% polyacrylamide gels, and the gels were stained with Coomassie Blue. The labeled poly(Glu/Tyr) was excised from the gel, and radioactivity was counted.

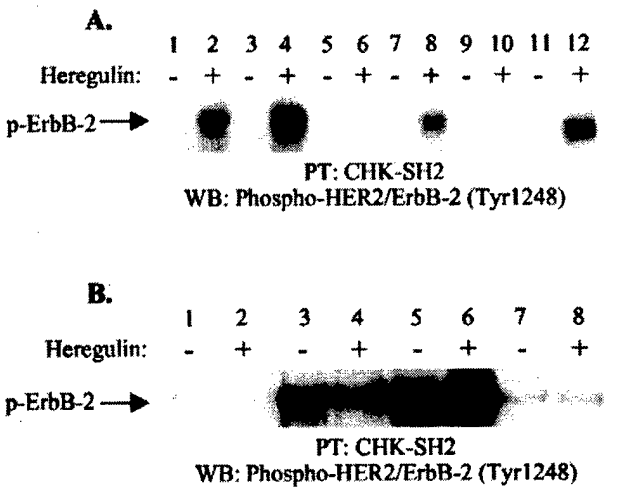

FIG. 2. **GST pull-down experiment.** Serum-starved T47D (*A*) or BT474 (*B*) cells were stimulated with 20 nM heregulin for 8 min at room temperature and then lysed in protein lysis buffer. Lysates were incubated with GST fusion proteins coupled to glutathione-Sepharose beads. The precipitated proteins were developed on an SDS-7% polyacrylamide gel and then transferred onto Immobilon-PM membranes. Bound proteins were immunoblotted with anti-phospho-HER2/ErbB-2 (Tyr^{1248}) antibody. *A*: *lanes 1* and *2*, wild-type CHK SH2 domain; *lanes 3* and *4*, G129R; *lanes 5* and *6*, R147A; *lanes 7* and *8*, R147K; *lanes 9* and *10*, empty GST beads; *lanes 11* and *12*, total cell lysate. *B*: *lanes 1* and *2*, empty GST beads; *lanes 3* and *4*, wild-type CHK SH2 domain; *lanes 5* and *6*, G129R; *lanes 7* and *8*, R147A. *PT*, precipitation; *WB*, Western blot.

RESULTS

Generation of the Binding Mutants

Two arginine residues are conserved in the Tyr(P)-binding pocket. Alignment of the CHK SH2 domain with other SH2 domain sequences shows that whereas the arginine in βB5 is present in these SH2 sequences, the αA2 CHK SH2 domain has a glycine. This arginine in the binding pocket of other SH2 domains provides a positive charge to coordinate the phosphate of Tyr(P), implying either a different mode of binding or weak binding for CHK. Arg^{147} in βB is a critical residue for Tyr(P) binding and is strictly conserved in SH2 domains (23). Based on these observations, we constructed three CHK SH2 domain mutants: G129R, R147A, and R147K. The G129R mutant encodes a CHK SH2 domain protein in which the αA2 glycine at the phosphotyrosine-binding pocket has been replaced by arginine. For the R147A and R147K mutants, the βB5 arginine has been replaced by alanine and lysine, respectively. Our hypothesis is that G129R will have enhanced ErbB-2 phosphopeptide binding, R147A will have disrupted binding, and R147K will have reduced binding.

Binding Studies

GST Pull-down Experiment—We conducted binding studies with the three CHK SH2 domain mutants as well as with wild-type CHK (Fig. 2). Two different breast cancer cell lines, T47D (moderate level of ErbB-2/neu expression) and BT474 (high level of expression), were tested. The conserved tyrosine residues of overexpressed ErbB-2/neu in BT474 cells were found to be autophosphorylated in the absence of ligand stimulation. The cells were serum-starved as described under "Experimental Procedures" and then activated with heregulin (20 nM) for 8 min. Unstimulated and stimulated cells were lysed and precipitated with GST-CHK SH2 fusion proteins as well as with GST protein alone. The precipitates were analyzed by SDS-PAGE and immunoblotted with anti-phospho-HER2/ErbB-2 (Tyr^{1248}) antibody. In comparison with wild-type CHK,

the substitution of Gly^{129} with Arg^{129} resulted in dramatically increased binding in both cell lines. In T47D cells (Fig. 2*A*), only heregulin stimulation induced the association of ErbB-2 with the purified wild-type SH2 domain and also G129R, indicating that G129R binding is ligand-stimulated to a similar level compared with wild-type binding. The substitution of Arg^{147} with Lys^{147} slightly decreased binding, whereas the substitution with Ala^{147} completely disrupted binding. These results indicate that the positive charge of Arg^{147} is critical for phosphopeptide binding and that the length of the side chain has a moderate effect on binding. Because the R147K mutant showed only a moderate effect on binding in comparison with the wild-type SH2 domain, the R147K mutant was not analyzed in further experiments. In BT474 cells, no ligand stimulation was necessary for the CHK SH2 domain interaction with the constitutively phosphorylated ErbB-2/neu protein (Fig. 2*B*). Again, almost no association of R147A with ErbB-2/neu was seen, whereas G129R pulled down markedly more ErbB-2 protein than did the wild-type CHK SH2 domain.

NMR Experiments—The backbone atoms of the CHK SH2 domain were assigned using triple resonance experiments (Fig. 3*A*). NMR, which is a method used to determine the high resolution structure of macromolecules, is particularly valuable in providing rapid identification of a ligand-binding site. The ^{15}N-^{1}H HSQC experiment yields a well resolved spectrum, with single peaks for the backbone amides of most residues in the protein (25). Changes in the positions of these peaks upon titration of the ligand can identify the residues in the binding site, and analysis of the titration data can be used to determine the kinetics of binding (24).

To analyze the interaction between CHK SH2 domain constructs and the phosphopeptide, we titrated the peptide into 0.6 mM ^{15}N-labeled SH2 domains. Progressive changes in ^{1}H and ^{15}N chemical shifts were monitored with a series of ^{15}N HSQC spectra. Significant chemical shift changes in the wild-type SH2 domain were observed for several residues in the αA, βB, and βD secondary structures as well as in the βD-αB and αB-βE loops (Fig. 3*A*), which have been implicated in the binding of other SH2 domains to phosphopeptides (26). In particular, several positive residues in βD (His^{168} and Arg^{170}) undergo large chemical shift changes upon complex formation, consistent with the positive charged residues in these secondary structural elements forming contacts with Tyr(P) of the ligand. In the αA helix, several residues changed chemical shifts upon complex formation (Ile^{127}, Gly^{129}, Gln^{134}, and Gln^{135}). The βB-βC loop has also been implicated in the binding of the peptide, and significant chemical shift changes were observed for residues in this loop (Ser^{149}, Arg^{151}, and Gly^{154}). Interestingly, Arg^{147} showed little change in chemical shift despite its presumed role in contacting the phosphate of the ligand. Significant chemical shift changes were also observed in residues in βD (Tyr^{169}, Val^{171}, and Leu^{172}), the βD-αB loop (Ile^{180} and Asp^{181}), and the αB-βE loop (Ile^{203}), which presumably form the hydrophobic environment for the hydrophobic residues of the C terminus to the Tyr(P) region of the peptide (see Fig. 5).

G129R, which showed enhanced binding, was also titrated with the phosphopeptide (Fig. 3*B*). The Gly^{129} resonance was replaced with a new resonance (Arg^{129}), consistent with the substitution of glycine with arginine and the expected changes in chemical shift (27). Several residues showed chemical shift changes upon mutation. In particular, residues in αA (Ile^{127}, Glu^{131}, Ala^{132}, and Gln^{134}) experienced chemical shift changes (0.3 ± 0.1 ppm) presumably due to introduction of the long charged side chain of Arg. Several residues in βB and βD also showed chemical shift changes, suggesting that they are in proximity to the G129R mutation site. Upon titration with peptide, residues similar to the wild-type residues underwent significant chemical shift changes, except for residues near the mutation site in αA and βB (Table I).

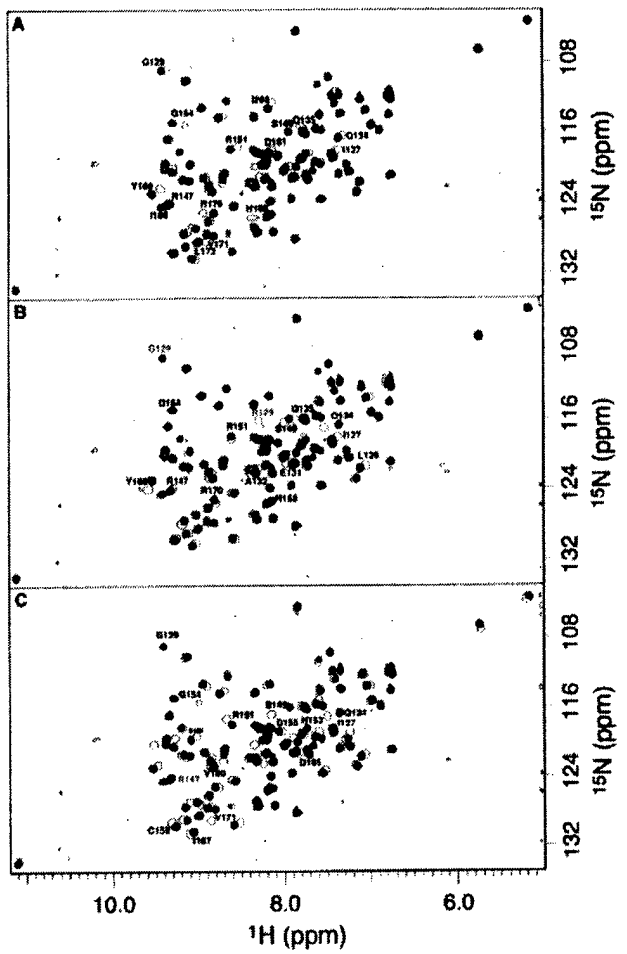

FIG. 3. *A*, superimposed ^{15}N-^{1}H HSQC spectra of the wild-type CHK SH2 domain without (*black*) and with (*red*) 1 eq of the Tyr(P) peptide (Ac-ENPEpYLGLDV-NH_2). *B*, superimposed ^{15}N-^{1}H HSQC spectra of the wild-type CHK SH2 domain (*black*) and the G129R mutant (*red*). *C*, superimposed ^{15}N-^{1}H HSQC spectra of the wild-type CHK SH2 domain (*black*) and the R147A mutant (*red*). The 500-MHz spectra were collected at 25 °C from samples containing ~0.6 mM SH2 domain. Peaks that significantly changed chemical shifts upon binding the peptide (*A*) or upon mutation (*B* and *C*) are labeled in *black* by residue number. Residues targeted for mutation are labeled in *red*.

R147A did not show chemical shift changes with any residue upon peptide titration, consistent with the complete disruption of binding seen in the GST pull-down experiment. With this mutation, the peak for Arg^{147} disappeared, but a new Ala^{147} peak was difficult to identify, presumably due to resonance overlap (Fig. 3*C*). Compared with G129R, the R147A substitution showed different chemical shifts for residues in regions such as αA (Ile^{127}, Ser^{128}, and Gly^{129}), βB (Ser^{149} and His^{152}), the βB-βC loop (Gly^{154} and Asp^{155}), βC (Cys^{159} and Val^{160}), and βD (Asp^{165}, Ile^{167}, and Val^{171}). In addition, Gly^{129} disappeared with the mutation, implying line broadening consistent with the introduction of a motion on the millisecond-to-microsecond time scale. Although both mutants showed perturbation at several residues, the overall structure seemed to be unaltered, as the majority of the residues showed little change (Fig. 3).

Equilibrium dissociation constants (K_d) were determined from a plot of chemical shift changes *versus* the ratio of peptide to protein. Chemical shifts of residues Ile^{127} (βA-αA loop);

TABLE I
Equilibrium dissociation constants and chemical shift changes of the wild-type SH2 domain and G129R

Residue[a]	Wild-type SH2		G129R	
	K_d	Shift[b]	K_d	Shift[b]
	mM	ppm	mM	ppm
Ile^{127}	ND	ND	0.41 ± 0.27	0.19 ± 0.04
Gly^{129}	1.1 ± 0.7	0.18 ± 0.06	ND	ND
Arg^{147}	ND	ND	0.68 ± 0.50	0.09 ± 0.03
Gly^{154}	0.74 ± 0.28	0.52 ± 0.09	0.09 ± 0.03	0.68 ± 0.50
Tyr^{156}	0.63 ± 0.14	0.33 ± 0.03	0.05 ± 0.05	0.20 ± 0.03
His^{168}	0.29 ± 0.04	0.62 ± 0.03	ND	ND
Tyr^{169}	0.27 ± 0.07	0.42 ± 0.03	0.27 ± 0.07	0.42 ± 0.03
Arg^{170}	0.23 ± 0.05	0.25 ± 0.01	0.26 ± 0.10	0.20 ± 0.02
Ile^{203}	1.89 ± 0.96	0.68 ± 0.22	0.15 ± 0.17	0.20 ± 0.03
Average	0.48 ± 0.05	ND	0.13 ± 0.08	ND

[a] These residues were selected because they were well resolved during peptide titration. ND, not determined because chemical shift differences were too small (Ile^{127}, Gly^{129}, and Arg^{147}) or because of spectral overlap (His^{168}).

[b] The chemical shift difference was calculated from the absolute value of the change in ^1H chemical shift plus 0.2 times the absolute value of the change in ^{15}N chemical shift.

Gly^{129} (αA); Arg^{147} (βB); Gly^{154} (βB-βC loop); Tyr^{156} (βC); His^{168}, Tyr^{169}, and Arg^{170} (βD); and Ile^{203} (αB-βE loop) were monitored because these residues were involved in the phosphopeptide interaction and remained well resolved throughout the titration. Consistent with the results from the GST pull-down binding assay, overall, wild-type CHK has a K_d of ~0.5 mM, showing five times weaker affinity than G129R (Table I). In wild-type CHK, residues in βD (His^{168}, Tyr^{169}, and Arg^{170}) showed tighter binding than Gly^{154}, Tyr^{156}, and Ile^{203}, which are predicted to be the C-terminal residues of the bound ErbB-2 phosphopeptide. In G129R, the affinity of Gly^{154}, Tyr^{156}, and Ile^{203} increased >10 times over that seen in wild-type CHK, whereas the residues in βD (His^{168}, Tyr^{169}, and Arg^{170}) remained the same as observed in wild-type CHK.

Src Kinase Assay

To investigate the functional role of the association of CHK and ErbB-2, we tested the effects of transient transfection with CHK-pIRES2-EGFP constructs on heregulin-stimulated Src kinase activity in MCF-7 cells. The expression levels of all studied forms of the CHK protein were similar as assessed by Western blotting (Fig. 4). As shown in Fig. 4, stimulation of MCF-7 cells with heregulin caused a dramatic increase in the activity of Src kinase. Transfection with the wild-type CHK gene strongly inhibited the heregulin-stimulated Src activity, whereas no significant difference was seen in cells transfected with the kinase-dead mutant of CHK or an empty vector control. Furthermore, the inhibition of heregulin-stimulated Src kinase activity was markedly diminished by R147A, whereas the G129R-mediated inhibition was somewhat stronger compared with wild-type CHK.

DISCUSSION

The interaction of SH2 domains and their phosphotyrosine-containing binding partners has been extensively studied because of its critical role in signal transduction and cancer development (23, 28). Studying the binding of the CHK SH2 domain to the ErbB-2 receptor is particularly important for understanding the development of breast cancer (17). Studies using phosphopeptide libraries to probe sequence specificity showed that there are two types of SH2 domains, types I and II (23). Type I SH2 domains involve non-receptor tyrosine kinases such as Src, Lck, Fyn, and Abl and exhibit preferences for the motif Tyr(P)-hydrophilic-hydrophilic-(Ile/Pro) (29). Type II SH2 domains include phospholipase Cγ1, Syp tyrosine phosphatase,

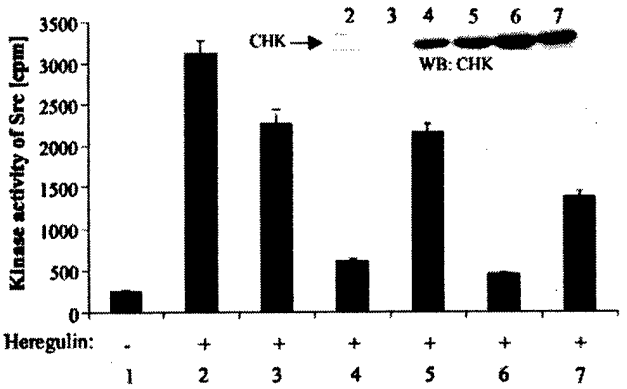

FIG. 4. **Src kinase assay.** MCF-7 cells were transiently transfected with 10 μg of various forms of CHK. The serum-starved cells were then stimulated with 20 nM heregulin for 8 min at room temperature, and total protein extracts were prepared. One milligram of protein was immunoprecipitated using antibodies against Src. The tyrosine kinase assay was performed as described under "Experimental Procedures." *Lanes/bars 1* and *2*, non-transfected MCF-7 cells; *lanes/bars 3*, empty vector; *lanes/bars 4*, wild-type CHK; *lanes/bars 5*, kinase-dead CHK; *lanes/bars 6*, G129R; *lanes/bars 7*, R147A. *Inset*, expression of various CHK proteins in transfected cells was assessed by Western blot (*WB*) analysis.

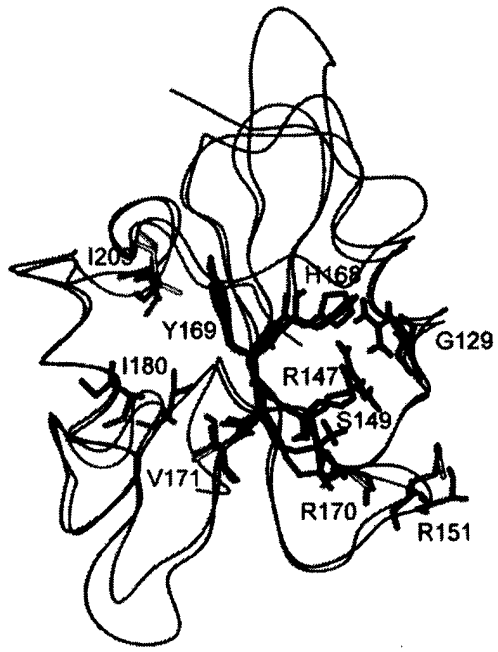

FIG. 5. **Superposition of the phosphopeptide-binding site of the CHK SH2 domain (*red*; Protein Data Bank code 1JWO) and the v-Src SH2 domain (*blue*; Protein Data Bank code 1BKL).** Residues involved in binding Tyr(P) (Gly^{129}, Arg^{147}, His^{168}, and Arg^{170}) are indicated in *red* for CHK and in *blue* for the corresponding residues of Src. Residues composing the hydrophobic site that interact with residues composing the C terminus to the Tyr(P) region of the phosphopeptide are indicated in *pink* (CHK) and in *green* (Src). The nonconserved Arg^{151} of CHK is indicated in *pink*.

Shc, and Grb2 and prefer the motif Tyr(P)-hydrophobic-*X*-hydrophobic. Structural analysis of the type I high affinity binding (10^{-8} M) SH2 domain-peptide complex showed that Tyr(P) (in pYEEI) is inserted into a large positively charged pocket and Ile into a smaller hydrophobic pocket. In contrast, the type II phospholipase Cγ1-peptide complex has relatively lower affinity binding ($K_d = 10^{-6}$ M) and demonstrates more extensive peptide interactions, using a long hydrophobic groove to accommodate the two hydrophobic residues in the peptide.

The CHK SH2 domain binds to the ErbB-2 phosphopeptide (pYLGLDV), which contains the consensus sequence for binding to a type II SH2 domain (30). HSQC experiments identified the residues involved in phosphopeptide binding. Residues of the positively charged Tyr(P)-binding site of CHK, as observed in other SH2 domain-peptide interactions, are predicted to be Gly^{129} in αA, Arg^{147} in βB, and His^{168} and Arg^{170} in βD. Residues of this binding site participate in electrostatic interactions with the Tyr(P) ring. The hydrophobic residues in the specificity-determining site (βD, βD-αB loop, and αB-βE loop) provide extensive hydrophobic contacts between the C-terminal hydrophobic residues and Tyr(P) of the peptide. Recently, the crystal structure of the CHK SH2 domain has been solved by Murthy *et al.* (34) (Protein Data Bank code 1JWO). The overall folding of the CHK SH2 domain is similar to that of other SH2 domains. Superposition of backbone atoms of the secondary structure residues of the CHK and Src SH2 domains showed that the overall folding and binding pocket are conserved (Fig. 5). The side chain of the nonconserved Arg^{151} of the CHK SH2 domain is positioned near the Tyr(P)-binding site. The positive charge of this residue may also contribute to binding. The chemical shift of Arg^{151} was changed by peptide addition.

The dissociation constant of the wild-type CHK SH2 domain was found to be 0.5 mM, which is much weaker than that of the low affinity type II SH2 domain (23). The low affinity for the phosphopeptide does not necessarily mean a low affinity of ErbB-2/neu for CHK *in vivo* in cells. Because $Tyr(P)^{1248}$ is the most critical residue in ErbB-2/neu-mediated cancer development, dynamic regulation can be involved in its interaction. We suspect that the unusual presence of Gly^{129} in the Tyr(P)-binding site is one reason for this weak binding. The GST pull-down experiment showed a dramatic enhancement of the binding when Gly^{129} was substituted with Arg^{129}. The K_d of G129R decreased by >5-fold, as determined from the NMR titration experiments. Both experiments indicated that G129R showed higher binding affinity for the ErbB-2 peptide. The similar chemical shift changes of the residues in the specificity-determining site of G129R and the wild-type SH2 domain indicate no alteration in specificity. Interestingly, the increased affinity of G129R is caused by a dramatic increase in the binding affinity of residues that are likely to bind to the C-terminal residues of the ErbB-2 phosphopeptide (Table I).

The R147K mutation decreased binding, whereas the R147A mutation completely disrupted binding. This result implies that the positive charge of Arg^{147} is critical to coordinate the negative charge of Tyr(P) and that the length of the side chain also affects the binding. The importance of Arg^{147} in SH2 domains has been tested in different systems, and the results indicate that this residue is critical for binding (31, 32). Alanine mutation of this strictly conserved Arg^{147} (βB) resulted in a large increase in ΔG_0 ($\Delta\Delta G_0$ = 3.2 kcal/mol), whereas mutations of other residues each resulted in a significantly smaller ($\Delta\Delta G_0 < 1.4$ kcal/mol) reduction in affinity. This indicates that Arg^{147} (βB) is an important determinant of the Tyr(P)-binding recognition site (33).

How can CHK be involved in negative growth regulation in human breast cancer? How can the specific binding of CHK to the most critical and strictly conserved autophosphorylation site ($Tyr(P)^{1248}$) of ErbB-2 be related to the down-regulation of ErbB-2-mediated Src family kinase activity? One model is that upon heregulin stimulation, CHK association with the ErbB-2 receptor locates the CHK near the substrate, Src, thereby causing growth inhibitory effects (13, 14). The enhanced binding or disrupted binding mutants were used to test whether the specific association of CHK and ErbB-2 is related to Src kinase activity. The R147A mutant markedly diminished the inhibition of Src activity, whereas the G129R mutant inhibited Src activity more potently in comparison with the wild-type SH2 domain. These results prove that the specific interaction of CHK and ErbB-2 via the SH2 domain is directly related to the growth inhibitory effects of CHK.

Because CHK shows restricted expression and specifically inhibits breast cancer development, CHK can be a potential candidate for gene therapy. However, the expression levels of CHK are very low in breast tumors, and CHK barely shows kinase activity in breast cancer tissues as compared with Csk (13–16). Because highly effective inhibition of Src activity is important for gene therapy, improved expression of the CHK gene, development of CHK with higher kinase activity, and construction of a higher affinity CHK SH2 domain would be very important for inhibiting breast cancer growth. Thus, the enhanced binding mutant (G129R) is promising as a potential candidate for gene therapy.

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